PRODUCTION OF CHITOSANASE BY RECOMBINANT *STREPTOMYCES LIVIDANS* AND ENZYMATIC PREPARATION OF CHITOSAN OLIGOMERS

par

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ABSTRACT

Elicitors are molecules which can trigger phytoalexin and pathogenesis-related proteins biosynthesis in plants. Chitosan as an elicitor has important roles in the interaction between pathogenic fungi and plants. Chitosan can inhibit fungal DNA transcription into mRNA, and it can also trigger the specific defensive genes which encode for at least 20 kinds of proteins related to resistant mechanism in plants.

Chitosanase is an endoglycosidase that can hydrolyze chitosan into oligosaccharide fragments. Chitosan heptamer-more fractions showed maximal activity in both antifungal properties and induction of plant resistance. In this work, recombinant strains of *Streptomyces lividans* were used for production of chitosanase, then we used this enzyme to prepare the active chitosan oligomers and to test their antifungal properties and their ability to induce defensive responses in plants.

In order to produce large amounts of chitosanase, the chitosanase gene from *Streptomyces* N174 was cloned in the high copy-number vector pFD666 and transformed into protoplasts of *S. lividans* TK24 and 10-164. These recombinant

strains could produce chitosanase efficiently. The chitosanase activity could reach up to 95 units per millilitre of fermentation liquid on natural substrate (mycelium of *Mucor rouxii*). When DNA sequences were deleted in upstream or downstream of the chitosanase gene, chitosanase production became lower. Maybe, these sequences have some functions for the chitosanase gene expression and stability. In the chitosan medium, the chitosanase activity of the recombinant strains *S. lividans* TK24 decreased quickly after 3 days. We have shown that it related to the production of a specific proteolytic enzyme for chitosanase. On the contrary, the chitosanase activity could maintain high level after 3 days in *M. rouxii* mycelium as fermentation substrate. If *S. lividans* 10-164 was used as host for carrying the chitosanase gene, chitosanase activity reached high level after 2 days and the same level was maintained for a few days in D-glucosamine medium. The enzyme was recovered by polyacrylic acid precipitation. The enzyme prepared with this method has stable activity for long time.

Using chitosanase hydrolysis, the heptamer-more fractions were prepared. These chitosan oligomers could inhibit fungal growth and could induce the production of pathogenesis-related proteins such as ß-1,3-glucanase, chitinase and chitosanase by plants.

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LIST OF ABBREVIATIONS

act.: activity

BSA: Bovine Serum Albumin

C.A.: casamino acid

CHS: chitosanase

chs: chitosanase-encoding gene

DMSO: dimethylsulfoxide

EDTA: ethylenediaminetetra-acetic acid (disodium salt)

ferm.: fermentation

g: gram

GlcN: glucosamine

$(GlcN)_2$: chitobiose

$(GlcN)_3$: chitotriose

$(GlcN)_4$: chitotetraose

$(GlcN)_5$: chitopentaose

$(GlcN)_6$: chitohexaose

$(GlcN)_7$: chitoheptaose

GlcNAc: N-acetyl-glucosamine

HAc:	acetic acid
HCl:	chlorhydric acid
hr:	hour
Kb:	kilobase
kDa:	kilodalton
L:	liter
M:	molar
M.E.:	malt extract
mg:	milligram
min:	minute
ml:	milliliter
mM:	millimolar
mol:	mole
MOPS:	4-Morpholinepropanesulfonic acid
mpv:	milliliters of packed volume
MS:	minimal salts
mU:	milliunit
nm:	nanometer
NaAc:	sodium acetate

neo:	aminoglycoside resistance gene
O.D.:	optical density
PA:	polyacrylic acid
PAGE:	polyacrylamide gel electrophoresis
PDA:	potato dextrose agar
PMSF:	phenylmethyl sulfonyl fluoride
P.-R.:	pathogenesis-related
pro.:	protein
rpm:	rotations per minute
SDS:	sodium dodecyl sulfate
TSA:	Tryptic Soy Agar
TSB:	Tryptic Soy Broth
U:	unit
µg:	microgram
µl:	microliter
µm:	micrometer
µmol:	micromole

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CHAPTER 1

INTRODUCTION

Elicitors are molecules of microbial origin which trigger the production of phytoalexins and pathogenesis-related proteins. Elicitors act as a signal inducing plant resistant responses and regulating biochemical and physiological processes. Elicitors include: glucans, chitosan, glucoproteins, polysaccharides and fatty acids.

Chitosan is a polymer of ß-1,4-glucosamine. In fact, it is deacetylated chitin (Fig. 1). As a practical approach, the chitosan polymer is defined as containing glucosamine moieties partially N-acetylated, with the degree (percentage) of acetylation cited (Fenton and Eveleigh 1981). Chitosan was first identified as a minor component of cell walls of *Phycomyces blakesleeanus* (Kreger, 1954) and further investigation established its presence throughout the cell walls of Zygomycete fungi, and in the cell walls of other fungi such as *Agaricus*, *Puccinia*, *Fusarium* and *Saccharomyces* (Ouakfaoui and Asselin 1992). Chitosan is a component of the cell walls of many plants pathogenic fungi, but it is not a normal compound of plant tissues. However, most commercially available chitosan is obtained via alkaline deacetylation of shellfish (usually crab) chitin.

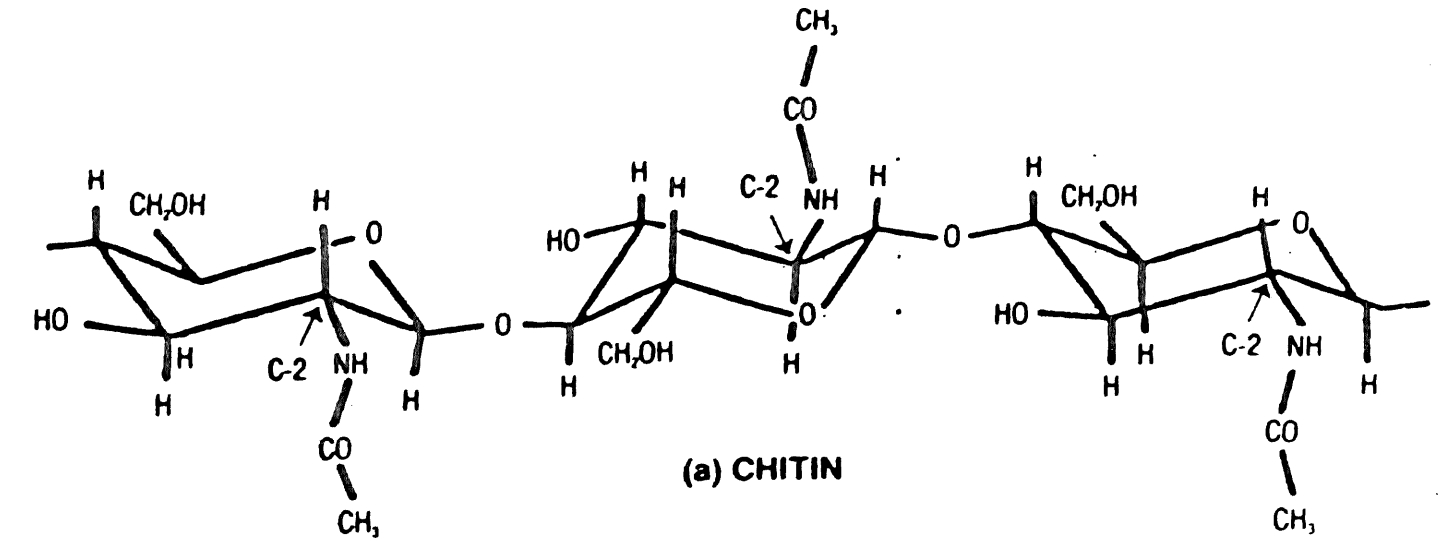


Fig. 1 Structure of chitin and chitosan

Chitosan as an elicitor has important roles in the interaction between plant and pathogenic fungi. For example, in the interaction between pea endocarp-tissue and *F. solani*, naturally released chitosan can accumulate in both the fungal cell and the adjacent host plant cells within 15 - 30 min, following inoculation of the fungi (Hadwiger and Beckman, 1980, 1981). Chitosan can inhibit germination of *F. solani* spores and growth of fungi at a concentration lower than 10 μg/ml. When chitosan was applied to pea tissue before the inoculation with *F. solani* f. sp. *pisi.*, chitosan induced defensive responses which developed a complete immunity of the pea to this pathogen. Chitosan can inhibit the fungal growth and induce the defensive responses of plant, because it has a strong affinity for DNA due to multiple positive charges (Hadwiger and Beckman, 1980) (Fig. 2). First, chitosan can inhibit fungal DNA transcription into mRNA, directly inhibiting the fungal growth. Second, chitosan can activate plant defense genes (Daniels et al., 1986) and induce the biosynthesis of at least 20 kinds of pathogenesis-related proteins, such as ß-1,3-glucanase, chitinase and chitosanase. These enzymes enhance plant potential to degrade the cell wall of fungi which results in the release of additional chitosan that can amplify the elicitor signalling process by feedback. In addition, chitosan induces the plant synthesis of phenylalanine ammonia lyase (PAL, a key enzyme in the phenylpropanoid pathway in plants).

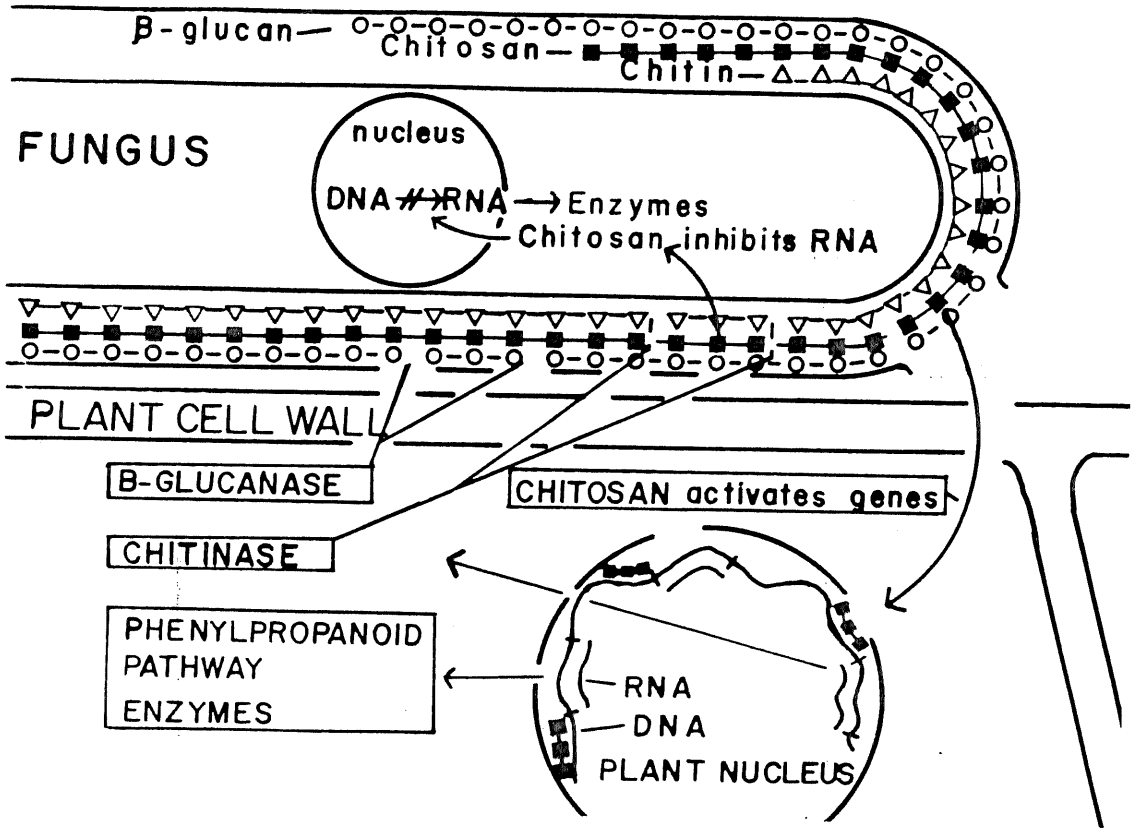


Fig. 2 Model of chitosan in plant-fungal pathogen interactions

Phytoalexins are produced by this pathway (Ryan, 1987; Loschke et al., 1983; Kendra and Hadwiger, 1984). Application of chitosan solution at a concentration of 100 μg/ml provides protection against *F. solani* f. sp. *pisi.* in pea pod tissue for periods of at least 5 days, while a concentration of 10 μg/ml can maintain resistance for up to 3 days (Kendra et al., 1989). There are also homolog of the resistance in potatoes (Matton and Brisson, 1989), soybean (Leguay et al., 1988), parsley (Somssich et al., 1988), birch, alder (Breiteneder et al., 1989) and bean (Walter et al., 1990).

The mechanism for triggering gene activation by chitosan is still not understood. Kendra and Hadwiger (1986) suggested that chitosan can directly interact with plant DNA to activate specific defense genes (Daniels et al., 1986). After an external application of chitosan, as well as cell wall components purified from the fungi *F. solani,* chitosan can be detected in the cytoplasm of pea cells within 20 min of application (Hadwiger and Loschke, 1981). This means that chitosan can penetrate the target cell plasma membrane very fast. This observation raises the possibility of an internal receptor different from the plasma membrane transduction system. Kendra and Hadwiger (1987) showed that there was no correlation between the chitosan or *F. solani* induction of disease resistant

responses in pea pod tissue and fluctuation in [Ca^{++}], inhibition of calmodulin, blockage of Ca^{++} channels or host membrane leakage. These results further indicated that cell wall or plasma membrane-bound Ca^{++} does not play a direct role in the chitosan activation of the pea resistance response mechanism. In contrast to these results, Young et al. (1982, 1983) have found that a linear correlation was found between calcium release from chitosan-treated whole cells or isolated cell wall and the amount of bound chitosan. The effect of chitosan on membrane permeability is due to its polycationic properties. Kohle et al. (1985) speculated that the Ca^{++} release after chitosan application may act as a secondary messenger to trigger host gene expression. However, most of the workers agree with Young et al. (1982). They showed that hyphal wall component can cause rapid depolarization of the transmembrane potential and may inhibit the electrogenic ion pump in the plasmalemme. Pelissier and Esquerre-Tugaye (1984) found that H^+ extrusion from plant cells is inhibited by fungal elicitors in plant and this effect is reversible. Recent data have shown that oligosacccharides cause the phosphorylation of a small protein in plasma membranes isolated from tomato and potato leaves (Ryan, 1988), and that a protein kinase seems to play an important role in the elicitation of the defense reaction in pea plant (Shiraishi et al., 1990). Verapamil, a Ca^{++} channel blocker, and K-252a, a strong inhibitor of protein

kinase, inhibit pisatin accumulation in pea epicotyl which had been treated with elicitor isolated from *M. pinodes*. Potential roles of Ca^{++}, cyclic AMP and phosphatidylinositide system in the induction resistance process need further investigations (Ryan, 1988; Shiraishi et al., 1990; Oku, 1992).

Chitosanase, a new class of enzyme, is an endoglycosidase that can hydrolyse chitosan into oligosaccharide fragments. The distinction between chitinase and chitosanase is not strict, as both enzymes have the ability to degrade a variety of chitosans with different degrees of acetylation (Ohtakara, 1988). The term chitosanase is usually given to enzymes having higher activity against highly deacetylated chitosan than chitin (Monaghan et al., 1973; Pelletier et al., 1990; Sakai et al., 1991). Chitosanases are produced by many micro-organisms, including fungi, bacteria and actinomycetes. In 1980, Hadwiger and Beckman reported that extracts of pea endocarp containing chitosanase could degrade *F. solani* f. sp. *pisi* and f. sp. *phaseoli* cell walls to produce carbohydrates fragments (chitosan and its oligomers) that have antifungal effect on *F. solani* and that act as powerful elicitors of the induction of pisatin (an isoflavinoid phytoalexin in the pods). Kendra and Hadwiger (1984) showed that monomer and dimer units have no antifungal activity but induce little pisatin production. Trimer through pentamer

units showed antifungal activity at high concentrations and have a moderate ability to induce pisatin formation. A sharp increase in antifungal activity and pisatin formation was noted for the hexamer units, while the heptamer and more fraction showed maximal activity in both inhibition of fungal growth and pisatin induction. These results indicated that the high-molecular-weight chitosan fragments are more active in both antifungal and pisatin formation activities than the intermediate and low-molecular-weight fragments. In practice, chitosan oligomers prepared by chitosanase hydrolysis are a mixture of oligomer fractions with different degree of polymerization. For economic reasons, a mixture of oligomers is used. Chitosan hydrolysate, which was prepared from chitosan by about 5% hydrolysis with chitosanase, has shown to be maximal in both antifungal and antibacterial activities (Table 1) (Uchida et al., 1989). This suggested that we should find the optimal degree of hydrolysis of chitosan in order to obtain a maximal effect both on antifungal activity and the induction of defensive responses in plants.

In order to produce chitosanase in large amounts, which could be used for the large scale preparation of the active chitosan oligomers, several chitosananolytic actinomycetes were isolated from soil by Brzezinski's research group (Dupuy, 1991). One of these strains, *Streptomyces* N174, was isolated from

Table 1 Minimum inhibitory concentration of chitosan and chitosan hydrolysate for fungi.

Fungi	Minimum inhibitory concentration (MIC), %		
	Chitosan	Chitosan hydrolyzate*	Chitosan oligomer I
Fusarium solani	0.07	0.035	NE
F. oxysporum	0.09	0.050	NE
F. oxysporum cepae	0.08	0.035	NE

* 50 mg total reducing sugar (TRS)/g of chitosan
NE = No effect at 1.0 %

a slightly acidic soil in a sugar maple grove near Sherbrooke (Fink et al., 1991; Boucher et al., 1992). This strain was highly effective in degradation of chitosan. The chitosanase gene from *Streptomyces* N174 was cloned in the high copy-number vector pFD666 (Fink et al., 1991) and several subclones were constructed (Masson et al., 1993; see also Fig. 3) and transformed into protoplasts of *S. lividans* TK24 and 10-164. Recombinant strains could produce chitosanase efficiently. This chitosanase was analyzed by SDS-PAGE. The molecular weight of the enzyme is 29.5 kDa; the isoelectric point is 7.5. The maximal activity of chitosan degradation was observed at 65°C, when the pH was maintained at 5.5. The K_m of the enzyme was 0.088 mg/ml and the V_{max} was 96.5 U/mg. The enzyme degraded chitosan with a range of acetylation degrees from 1 to 60% but not chitin or CM-cellulose. The enzyme showed an endo-splitting type activity and the end-product of chitosan degradation contained a mixture of dimers and trimers of D-glucosamine (Boucher et al., 1992). Here, I describe further studies on the production of chitosanase by recombinant *S. lividans* carrying the chitosanase gene from *Streptomyces* N174, and application of chitosanase to prepare the active chitosan oligomers as an elicitor.

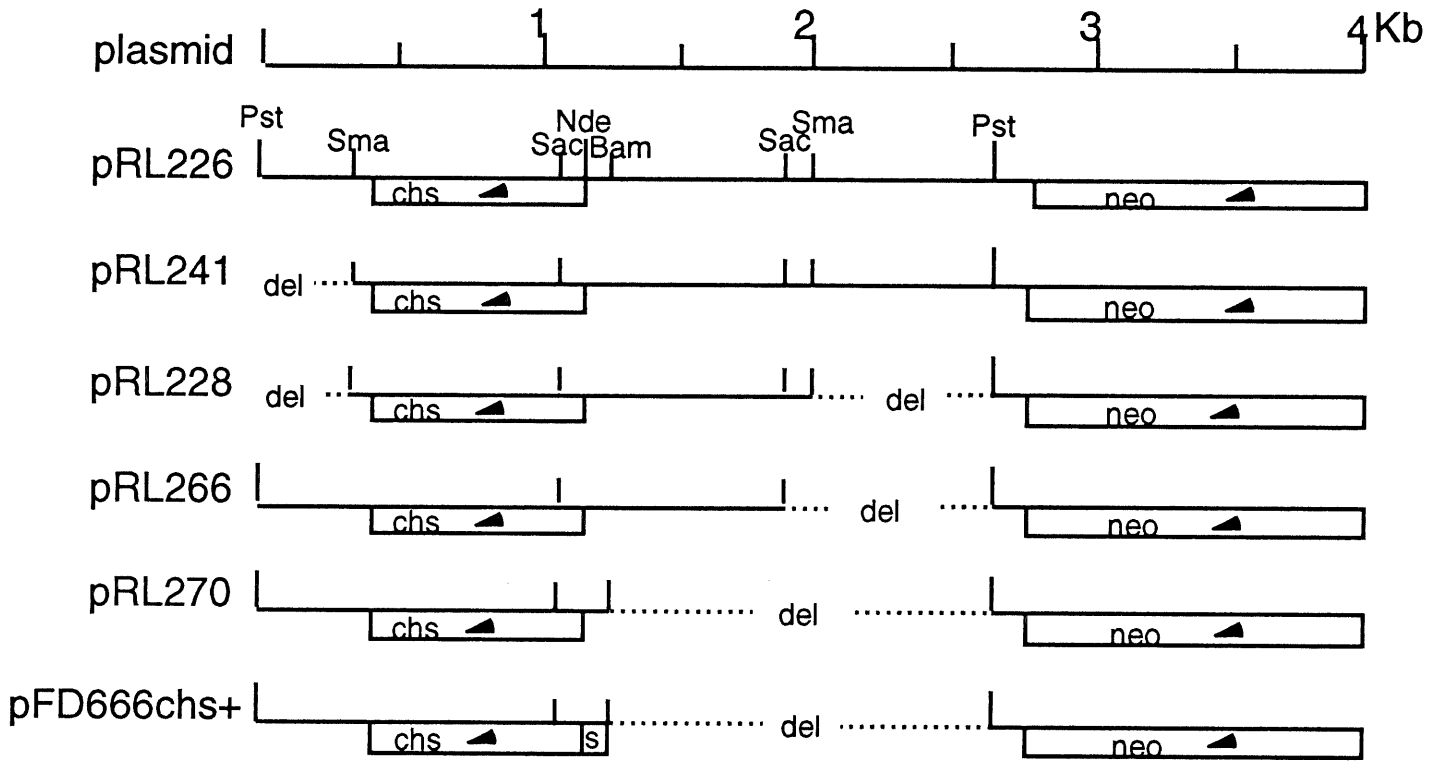


Fig. 3 The cloned chitosanase gene and its position in respect to the *neo* gene of the vector.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plants, microorganisms and plasmids

Plants, microorganisms and plasmids used in this study are listed in Table 2.

2.2 Media and culture conditions

2.2.1 *Streptomyces lividans*

The sporulation medium was SLM-3 with 50 μg/ml of Kanamycin (DeWitt, 1985). Spores were prepared from SLM-3 plates heavily inoculated with mycelial cultures and incubated for 2 weeks at 30°C.

Pre-culture medium was TSB containing 10 μg/ml of Kanamycin. The spores were directly taken from SLM-3 plates and were used to inoculate the TSB liquid medium. Culture was incubated 20-24 hr at 30°C on rotary shaker at 300 rpm, then the pre-culture was centrifuged and the volume of the pellet was

Table 2 The plants, organisms and plasmids used in this study.

Name	Source
Streptomyces lividans TK24	D. A. Hopwood John Innes Institute Norwich, England
Streptomyces lividans 10-164	Dieter Kluepfel Institut Armand-Frappier ville de Laval, Québec, Canada
Mucor rouxii	ATCC[1] #24905
Aspergillus niger	ATCC #336
Saccharomyces cerevisiae	ATCC #2360
Rhizopus oryzae	NRRL[2] #395
Fusarium oxysporum f.sp. *radicis-lycopersici*	Pierre Mathieu Charest Dept. de phytologie, F.S.A.A. Université de Laval Québec, Canada
pRL241 pRL226 pRL228 pRL266 pRL270	Jean-Yves Masson Isabelle Boucher Dept. de biologie Université de Sherbrooke Sherbrooke, Québec, Canada
pFD666 *chs+*	F. Denis Institut de Recherches Cliniques de Montréal
Tomato seeds	HYB. PERRON 2000 W.H.Perron
Tobacco seeds	XANTHI W.H.Perron

[1]) American Type Culture Collection

[2]) Northern Regional Research Laboratory, Peoria (IL) U.S.A.

measured (expressed as mpv). The pellet was washed with physiological salt solution water (NaCl 0.85%), and used for the inoculation of fermentation medium.

Fermentation medium contained the appropriate carbon source (at a concentration of 2% (w/v)) in minimal salts (MS) solution (Neugebauer et al, 1991). In some experiments, the nitrogen source was omitted from MS solution. Then 1mg/ml riboflavin and 1M MOPS (pH 7.0) were added to the medium after sterilization (riboflavin and MOPS final concentration was 1μl/ml and 0.1M respectively) (Boucher, 1992). The fermentation liquid media were inoculated at different densities with mycelium taken from the pre-culture of *S. lividans* and the cultures were incubated at 30°C on a rotary shaker at 300 rpm.

2.2.2 Fungus

Liquid medium contained malt extract (30g/L), proteose peptone (3g/L) and agar (15g/L). Fungi were incubated on rotary shaker at 120 rpm at 25°C.

Solid medium was potato dextrose agar (PDA). Antifungal properties of

chitosan oligomers were tested on PDA medium containing various concentrations of the chitosan oligomers. Fungus inoculum was set down in the centre of PDA Petri plate and incubated at 28°C.

2.2.3 Plants

Seeds were sterilized in 10% (v/v) bleach for 10 min and washed twice with sterile distilled water. Then, seeds were put on 0.6% agar Petri dishes mixed with various concentrations of chitosan oligomers for germination (Hirano et al., 1988). They were incubated at room temperature for 6 days.

Plants were grown in 50% vermiculite and 50% planta-mix soil, with a 16 hours photo-period in a growth chamber (23-25°C).

2.3 Enzymes assays

2.3.1 Standard substrates

Chitosanase: 250 mg of chitosan (practical grade C-0792, Sigma) was

dissolved in 25 ml of 1% HAc, then pH was adjusted at 5.5 with 50 mM sodium acetate and completed with distilled water in 100 ml. The final concentration of chitosan solution was 0.25%.

Chitinase: chitin (practical grade C-7170, Sigma) was suspended in 0.05 M McIlvaine buffer, pH 6.8. The concentration of chitin was 2%.

ß-1,3-glucanase: laminarin (L-9634, Sigma) was dissolved in 0.05 M McIlvaine buffer, pH 6.8. The concentration of laminarin was 2%.

Protease: azocasein (Sigma) was dissolved in 0.1 M Tris-Cl buffer, pH 8.0. The concentration of azocasein was 6 mg/ml.

2.3.2. Enzymatic reactions and measurements

Chitosanase: the chitosanase activity standard assay contained 950 μl of 0.25% chitosan substrate, 1-20 mU of enzyme and distilled water to a final volume of 1.0 ml. The reaction mixture was incubated in a water bath at 37°C for 10 min. The reaction was terminated by addition of 250 μl of 0.5 M NaOH. After keeping

the reaction tube in ice for 10 min, the reaction tube was centrifuged in order to eliminate the chitosan precipitate. Soluble reducing sugars were measured by Nelson-Somogyi assay (Spiro, 1966) as follows: 0.5 ml of supernatant was put into a tube containing 0.5 ml of alkaline copper reagent. After boiling 15 min in water bath, the reaction tube was chilled under tap water, then 0.5 ml of arsenomolybdate reagent and 4.5 ml of distilled water were added before centrifugation. Reducing sugars were measured spectrophotometrically at 520 nm.

Chitinase and ß-1,3-glucanase: the procedure was the same as for chitosanase, except that the reaction incubation time was 30 min.

Protease: the overall proteolytic activity standard assay contained 0.5 ml of substrate and 0.5 ml of the respective enzyme crude extract. After 30 min incubation at 50°C, undigested substrate was precipitated by adding 0.5 ml 10% trichloroacetic acid (TCA). The absorbency of the supernatant was determined at 366nm after centrifugation.

2.3.3 Definition of enzyme activity unit

One unit (U) of chitosanase activity was defined as the amount of enzyme that liberated 1 μmol equivalent D-glucosamine in 1 min under the above conditions.

One unit (U) of chitinase activity was defined as the amount of enzyme that liberated 1 μmol equivalent N-acetyl-glucosamine in 1 min under the above conditions.

One unit (U) of ß-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 μmol equivalent glucose in 1 min under the above conditions.

One unit of protease activity was defined as the amount of enzyme required to increase the $O.D._{366}$ of 0.01 under above conditions.

2.4 Total proteins determination

The protein determination was done according to Bradford's method modified by Stoscheck (Bradford, 1976; Stoscheck, 1990). Ten mg of Serva blue G (C.I.42655, Serva) was dissolved in 10 ml of 85% phosphoric acid and 5 ml of 95% ethanol, the solution was then diluted to 100 ml with distilled water, filtrated on two layer of S&S filter paper #410, and stored at 4°C. Seventy-five μl of 1M NaOH was added to 1 ml of this stock reagent before mixing with the protein. The reactive solutions were gently mixed and incubated 5 min at room temperature. Optical density at 590 nm was measured. The results were compared with the BSA standard curves in low absorbance level (O.D. and μg protein in linear relation), and high absorbance level (log O.D. and log μg protein in linear relation) (Stoscheck, 1990).

2.5 SDS-polyacrylamide gel electrophoresis

Samples were analyzed by SDS-PAGE (12% acrylamide). The electrophoresis conditions were described by Laemmli (1970). The gel was stained with Coomassie Blue (Sambrook et al., 1989).

2.6 Preparation of the active chitosan oligomers by chitosanase hydrolysis

2.6.1 Preparation of the chitosan oligomers

The procedure of preparation of the chitosan oligomers by chitosanase hydrolysis was done as follows: 2 grams of chitosan were dissolved in 1% acetic acid and pH was adjusted to 5.5 with ammonium hydroxide. The chitosan solution was divided into eight tubes (each tube containing 250 mg chitosan). Various amounts of chitosanase were added in each tube: 0.5U, 1.0U, 1.5U, 2.0U, 3.0U, 4.0U, 5.0U and 6.0U (for optimising the units of chitosanase). After mixing, tubes were incubated at 37°C in a water bath for 10 min. The reaction was terminated by boiling 20 min. Tubes were cooled down at room temperature. Then two volume of 95% ethanol were added dropwise, tubes were mixed gently and put on table until flocculation occurred. After 30 min centrifugation, the pellet was taken and washed twice with 95% ethanol to eliminate HAc (ethanol should be added 2 or 3 times volume of pellet). The pellet was washed with ether twice again and dried at room temperature. The dry weight was determined.

2.6.2 Testing the chitosan oligomers by thin-layer chromatography

The chitosan oligomers were analyzed by thin-layer chromatography (silica gel Whatman AL SIL G/UV). The developing solvent was a solution containing n-propanol, distilled water and ammonium hydroxide (70:30:1). The staining solution contained p-anisaldehyde, 95% ethanol, concentrated sulfuric acid and glacial acetic acid (1:18:1:0.2). Sugars were visualized by spraying staining solution and incubating at 95°C for about 5 min (Chaplin, 1986). The standard used were 14 mM GlcN, 14 mM GlcNAc, 10 mM $(GlcNAc)_2$, 30 mM $(GLcN)_3$ and 30 mM $(GLcN)_4$, (putting 5-10 μl of sample per lane).

2.7 Purification of chitosanase with polyacrylic acids

The procedure of precipitation of chitosanase from fermentation liquid with PA (Sternberg, 1976; Boucher et al., 1992) was done as follows: the chitosanase activity, the total proteins and the volume of the fermentation liquid supernatant were measured. The fermentation liquid supernatant was cooled down to 4°C (this temperature was maintained throughout the procedure) and the pH was adjusted down to 4.5 with 5 M HAc. The fermentation liquid was kept in ice bath and

mixed with a magnetic stirrer. A 4% (w/v) solution of PA was added drop by drop. The quantity of this solution which should be added was determined empirically (Boucher et al., 1992) as follows:

Volume (ml) of 4% PA solution =

Volume (ml) of ferm. liquid $\times$ concentration of proteins (μg/ml) $\times$ 10^{-4}

After mixing with a magnetic stirrer another 30 min, the precipitate was collected by centrifugation at 5000 rpm for 30 min, and the pellet was resuspended in 1/5 of the original volume of distilled water. NaOH (1 M) was added until the pH reached 8.5. To remove residual PA, calcium acetate solution (1 M) was added dropwise (the final concentration reached up to 35 mM) and mixed with a magnetic stirrer for 30 min again until calcium polyacrylate precipitated. The precipitate was removed by centrifugation. The supernatant (containing chitosanase) was acidified down to pH 5.0 with 1 M HAc. The chitosanase activity, the total proteins and the volume of the crude enzyme were measured again. This crude enzyme extract could be used directly or stored at -20°C after adding 1 volume of sterile glycerol.

2.8 Transformation of plasmids into *Streptomyces* protoplasts

The plasmids pRL226 and pRL270 were transformed into *S. lividans* 10-164 protoplasts following the procedure of Hopwood et al. (1985). Transformants were recovered on R2YE regeneration medium. After incubating at 30°C for 14-20 hrs, the plates were overlaid with soft agar containing Kanamycin (5 mg per plate) to select the resistant colonies.

2.9 Extraction of the crude enzymes from plants

2.9.1 Plant seedling extracts

Plant enzymes from seedlings were recovered according to the procedure of Hirano et al. (1988) modified as follows: whole seedlings were collected from agar plates and put in a mortar kept in ice bath. Ten volumes of 0.05 M potassium phosphate buffer solution (pH 6.8) were added to seedlings. The seedlings were homogenized with quartz sand. The plant seedlings debris were eliminated by centrifugation (5000 rpm, 20 min at 4°C). The supernatant was either immediately tested for enzymatic activities or frozen at -20°C until further analysis.

2.9.2 Plant leaves extracts

Intercellular fluids of plant leaves were extracted according to Parent and Asselin (1984) with the following modifications: freshly collected leaves were cut with scissor into pieces of 4 to 5 cm^2. Pieces were infiltrated *in vacuo*, with gentle agitation in a large excess of a cold (4°C) mixture: 0.05 M potassium phosphate buffer, pH 6.8, containing 0.5 mM PMSF (PMSF is dissolved in isopropanol) for at least three periods of 30 seconds. Leaves pieces were gently blotted until dry, rolled up and placed in a special tube setting shown on Fig. 4. The leaves intercellular fluid was collected by centrifuging at 1000 rpm for 15 min at 4°C in the small tube. It was either used immediately for enzymatic analysis or frozen at -20°C until further analysis.

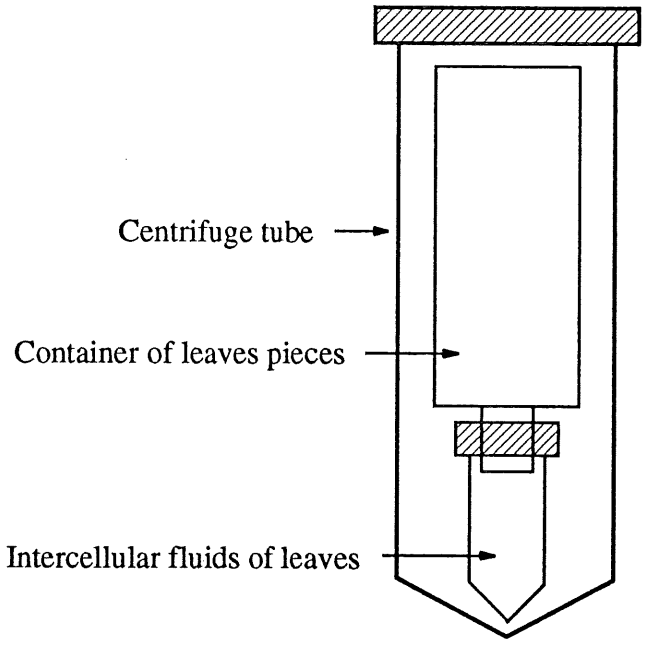


Fig. 5 Tube setting for the preparation of intercellular fluids of leaves.

CHAPTER 3

RESULTS

3.1 Optimization of the conditions for chitosanase production

Before producing large amounts of chitosanase, the optimal fermentation conditions for the chitosanase production by recombinant *S. lividans* had to be determined.

In preliminary experiments, the recombinant *S. lividans* TK24 (pRL226) was inoculated in different media, and the chitosanase activities were measured.

First, *S. lividans* TK24 (pRL226) spores (taken from SLM-3 plate) were inoculated directly into different fermentation media. The detected chitosanase activity was very low, less than 0.5 units per ml of fermentation liquid (Table 3), and the culture growth was not abundant. Probably, in fermentation media, spore germination is not easy. Consequently, a rich complex medium was used to help the spores germination as in experiment II.

In a second experiment, the spores were inoculated into TSB liquid medium

(containing 10 μg of Kanamycin per ml) for pre-culture before inoculating into the fermentation media. By this way, the chitosanase activities increased almost up to 20 times compared with the first experiment (Table 3).

From these preliminary experiments, we found that: first, it is very important to search the optimal fermentation condition of chitosanase gene expression and chitosanase production in recombinant *S. lividans*, because when we used different fermentation conditions we got very different activity level. Second, in order to approach the optimal fermentation conditions, it is necessary to pre-culture the spores in TSB medium for help spore germination (see section 4.2).

These results also led us to set up a new series of experiments to define the optimal fermentation conditions for maximal chitosanase gene expression.

Table 3 Preliminary experiments for chitosanase production by recombinant *S. lividans* TK24 (pRL226).

I. Spores inoculated directly into ferm. liquid			
Medium	Ferm. time* (days)	CHS act. (U/ml)	Specific act. (U/mg-pro.)
Chitosan 1.0%		0	
Casamino acid 1.0%	1	0.25	6.25
Chitosan 0.5% + Casamino acid 0.5%	1	0.28	4.31
Starch 1.0%	6	0.49	13.07
II. Spores were pre-cultured one-day in TSB medium before inoculation into ferm. liquid			
Chitosan		0	
Casamino acid 1.0%	1	0.51	12.75
Chitosan 0.5% + Casamino acid 0.5%	1	4.20	42.00
Starch 1.0%	9	7.90	77.07

*: The time taken for the chitosanase activity to reach the maximum.

**: The ferm. media use 0.05% asparagine as nitrogen source.

3.1.1 The optimal fermentation conditions of chitosanase production by recombinant *S. lividans* TK24 (pRL226)

Step 1. Effect of carbon source on chitosanase production

In order to find appropriate carbon source, chitosanase production was compared by *S. lividans* TK24 (pRL226) in fermentation media containing different carbon sources (or different combinations of carbon sources). The chitosanase activity and total proteins were measured in culture supernatant every day (Table 4 and Fig. 5).

From this experiment, it was found that chitosanase production was higher in the presence than that in the absence of chitosan in the fermentation media. It is possible that chitosan could act as an inducer of chitosanase gene expression (see section 4.3). However, when chitosan was used as the sole carbon source in the medium, the chitosanase gene could not be expressed. Probably, *S. lividans* growth is not easy in medium with chitosan as sole carbon source because *S. lividans* needs synthesis of other kinds of proteins before expression of the

Table 4 Effect of carbon source on chitosanase production

Carbon source in the medium	Ferm. time* (days)	CHS act. (U/ml)	Spcific act. (U/mg-pro.)
1. Chitosan 1.0%		0	
2. Chitosan 0.5% + Starch 0.5%	**2**	**16.00**	**53.69**
3. Chitosan 0.1% + Starch 0.9%	2	6.78	58.96
4. Starch 1.0%	8	7.46	43.25
5. Chitosan 0.5% + M.E. 0.5%	**2**	**15.00**	**45.39**
6. M.E. 1.0%	6	10.94	27.73
7. Chitosan 0.5% + C.A. 0.5%	1	4.20	42.00
8. C.A. 1.0%	1	0.51	12.74
9. Chitosan 0.5% + Chitin 0.5%		0	
10. Chitin 1.0%		0	

*: The time taken for the chitosanase activity to reach the maximum.

**: The strain used was *S.lividans* TK24 (pRL226).

***: The ferm. media used 0.05% asparagine as nitrogen source.

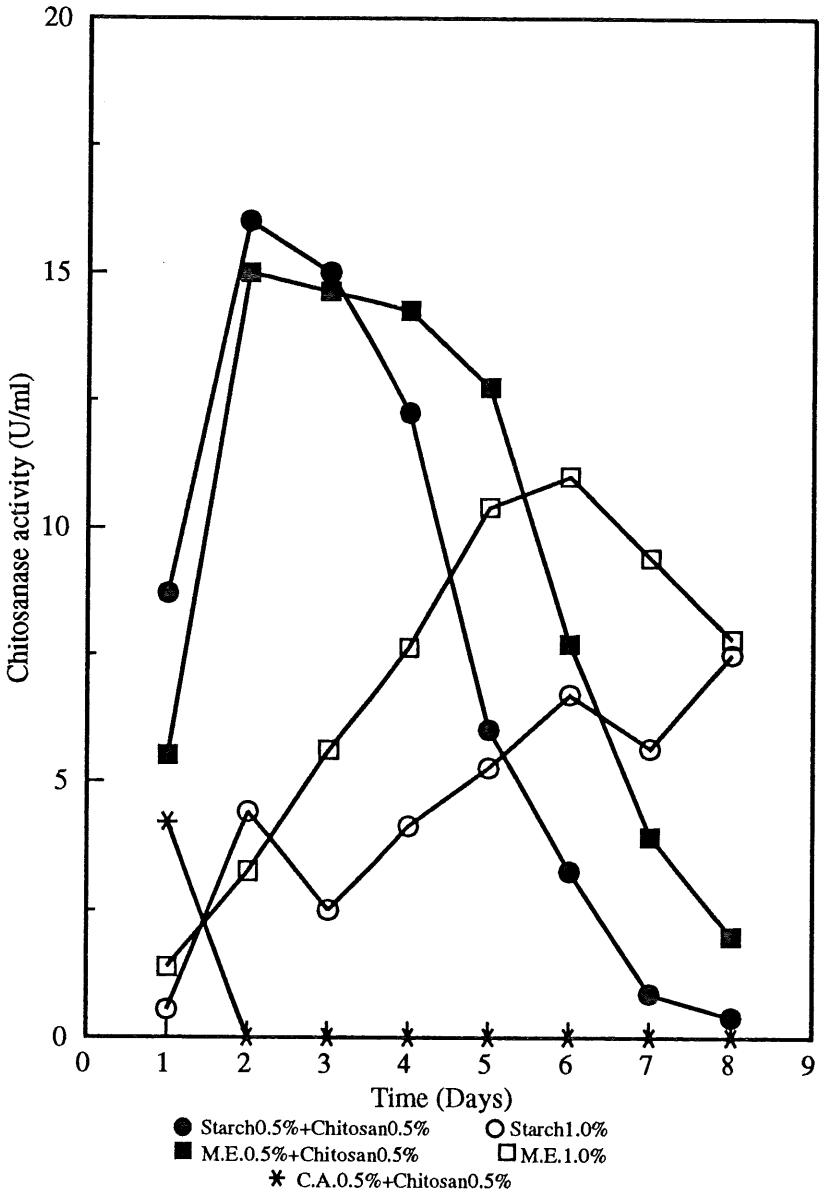


Fig. 5 Effect of carbon source on chitosanase production.

chitosanase gene.

After comparison, chitosan plus starch or malt extract media were chosen as the appropriate carbon sources for chitosanase production. Table 4 and Fig. 5 showed that more activity was produced in the chitosan-starch medium; but the activity was more stable in chitosan-malt extract medium (after reaching a maximum, the activity decreased slower).

Step 2. Optimization of the ratio between chitosan and enrichment

Table 4 showed that the chitosanase production was dependent on the ratio between chitosan and starch. Consequently, some additional experiments were carried out to determine the optimal ratio between chitosan and enrichment in fermentation medium. According to our results, 1.5% of chitosan plus 0.5% of enrichment as carbon source was the optimal ratio for getting maximal activity and higher specific activity (Table 5). Along with the decrease of chitosan ratio, the chitosanase production became lower. In 1.5% of chitosan plus 0.5% of malt extract fermentation medium, the chitosanase activity could reach up to 41.9 units per ml of fermentation liquid and the specific activity could reach up to 65.47 units

per mg of proteins (this value was very close to the purified chitosanase which had a specific activity of 66 units per mg of proteins) (Table 5).

Table 5 Optimization of the ratio between chitosan and enrichment.

No	Medium Chitosan% + M.E.%	Ferm. time* (days)	CHS act. (U/ml)	Specific act. (U/mg-pro.)
1.	**1.5 0.5**	**3**	**41.90**	**65.47**
2.	1.0 1.0	2	23.25	45.81
3.	0.5 1.5	5	29.75	44.07
4.	0.2 1.8	4	15.00	33.44
5.	0.0 2.0	6	12.00	33.48

* : The time taken for the chitosanase activity to reach the maximum.

** : The strain used was *S.lividans* TK24 (pRL226).

*** : The ferm. media used 0.05% asparagine as nitrogen source.

Step 3. Effect of nitrogen source on chitosanase production

In order to find a cheap and efficient nitrogen source, we tested chitosanase activity in media with different nitrogen sources. We found that 0.2% of ammonium sulphate was the best one (Table 6). It was cheaper and more efficient (four times) than asparagine which used before. When urea was used as nitrogen source, the chitosanase production could not be detected, even if *S. lividans* could grow in this medium. Using 0.2% of ammonium sulphate as nitrogen source in the fermentation medium, the chitosanase activity was 39.7 units per ml of fermentation liquid with a specific activity of 39.08 units per mg of proteins.

Step 4. Optimization of inoculum

We also analysed the effect of the quantitative inoculum on chitosanase production. To our surprise, the chitosanase activity greatly varied with the amount of inoculum (Table 7, Fig. 6). This difference was more than 20 units per ml of fermentation liquid. It indicated the importance of optimization of inoculum for chitosanase production. The results showed that addition of 4% mpv of pre-culture to the fermentation medium was the optimal quantitative inoculum for chitosanase

Table 6 Effect of nitrogen source on chitosanase production.

Nitrogen	Ferm. time* (days)	CHS act. (U/ml)	Specific act. (U/mg-pro.)
1. Asparagine 0.05%	2	9.52	56.00
2. Ammonium sulphate 0.2%	**4**	**39.70**	**39.08**
3. Urea 0.2%		0	
4. Corn steep liquor 1.0%	2	12.10	16.93

*: The time taken for the chitosanase activity to reach the maximum.

**: The strain used was *S. lividans* TK24 (pRL226).

***: The ferm. media was 1.5% chitosan+ 0.5% M. E.

Table 7 Optimization of inoculum.

No.	Inoculum % (mpv)	Ferm time* (days)	CHS act. (U/ml)	Specific act. (U/mg-pro.)
1	1.3	3	35.00	55.60
2	2.7	3	35.50	35.50
3	**4.0**	**3**	**59.50**	**52.42**
4	5.3	3	58.00	46.05
5	8.0	3	37.00	34.77

*: The time taken for the chitosanase activity to reach the maximum.

**: The strain used was *S. lividans* Tk24 (pRL226).

***: The ferm. media were 1.5% chitosan + 0.5% M. E., as well as 0.2% ammonium sulphate as nitrogen source.

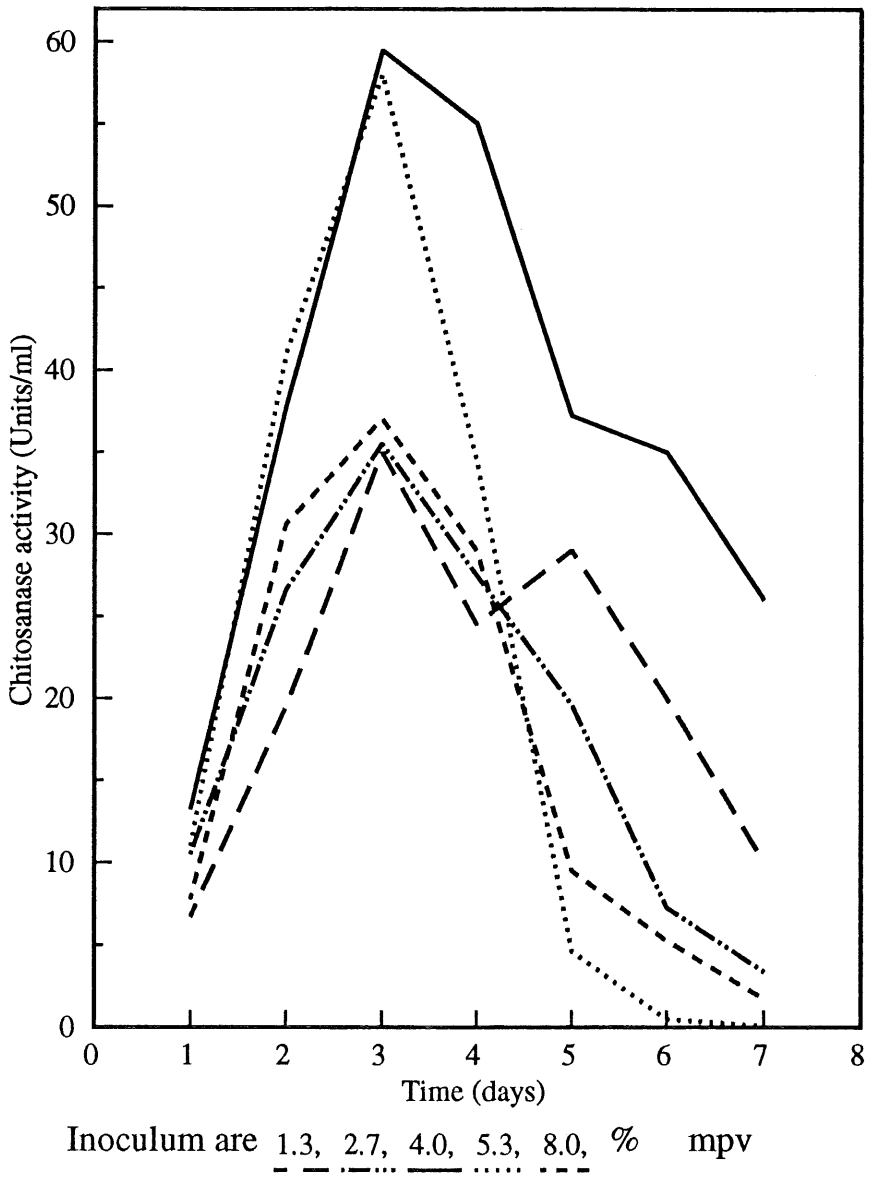


Fig. 6 Optimization of inoculum

production (Table 7, Fig.6). With this amount of inoculum, chitosanase activity could reach up to 59.5 units per ml of fermentation liquid with a specific activity of 52.42 units per mg of proteins.

Step 5. Chitosanase production on natural substrates

As previously mentioned, chitosan is a natural component of fungal cell walls. Fungal cell walls should be an economical fermentation substrate for chitosanase production. We used four kinds of sterile microorganism cells and mycelia instead of chitosan as substrate for fermenting recombinant *S. lividans*. We found that all of them could act as substrates for chitosanase production. Specially, it was observed that the chitosanase activity could be maintained at high level for a few days when *M. rouxii* mycelium was used as substrate. The activity reached up to 94.5 units per ml of fermentation liquid and the specific activity was 59.62 units per mg of proteins after 8 days. The same phenomenon was observed with *R. oryzae* mycelium as substrate. The activity could reach up to 62.5 units per ml of fermentation liquid after 9 days. But in chitosan substrate, the chitosanase activity reached only 59.5 units per ml of fermentation liquid after 3 days, then decreased quickly (Fig. 7, Table 8). These showed that fungal

mycelium could be a convenient substrate for large scale production of chitosanase.

Table 8 Chitosanase production on natural substrates.

Medium	Ferm. time* (days)	CHS act. (U/ml)	Specific act. (U/mg-pro.)
1. Chitosan 1.5% + M.E.0.5%	3	59.50	52.45
2. M.rouxii 1.5% + M.E.0.5%	**8**	**94.50**	**59.62**
3. M.rouxii 2.0%	**8**	**90.00**	**51.43**
4. M.rouxii 2.0% (-NH_4^+)**	6	21.25	51.70
5. A.niger 1.5% + M.E. 0.5%	5	29.00	46.07
6. S.cerevisiae1.5% + M.E.0.5%	3	28.25	28.01
7. R. oryzae 2.0%	9	62.50	46.33

*: The time taken for the chitosanase activity to reach the maximum.

**: There was not nitrogen source in MS solution.

***: The strain used was *S. lividans* TK24 (pRL226).

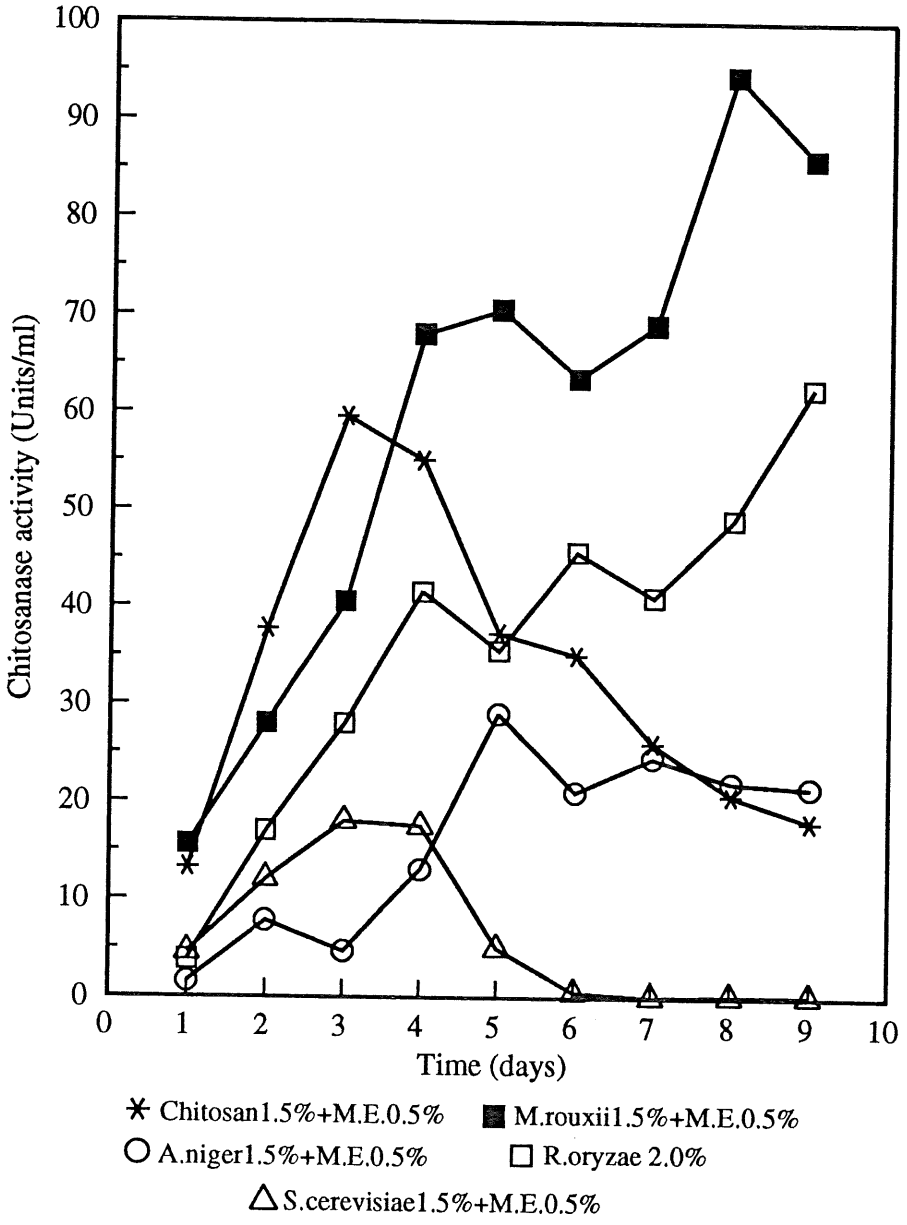


Fig. 7 Chitosanase production on natural substrates.

Conclusion

We can conclude that the chitosanase production was very efficient in chitosan medium enriched with starch or malt extract, but the highest chitosanase activity level was obtained in medium containing *M.rouxii* mycelium instead of chitosan. The chitosanase activity could reach up to 94.5 units per ml of fermentation liquid with the specific activity of 59.62 units per mg of proteins.

3.1.2 Comparison between different clones carrying the chitosanase gene

The chitosanase gene from *Streptomyces* N174 was cloned into the vector pFD666 at PstI site, as plasmid pRL226. By deleting some DNA sequences in upstream or downstream of the chitosanase gene, several other derivatives were constructed: pRL241, pRL228, pRL266 and pRL270 (Masson et al., 1993; see also Fig. 3). Furthermore, to test the chitosanase gene expression driven by the synthetic consensus promoter (Denis and Brzezinski, 1991), we used a clone in which the native chitosanase gene promoter was replaced with the synthetic consensus promoter: the clone pFD666 *chs*+ (obtained from Dr. F. Denis, Institut

de Recherches Cliniques de Montréal). The restriction maps of these plasmids are shown in Fig. 3. The plasmids were transformed into *S.lividans* TK24. When we inoculated these recombinant strains in the optimal fermentation conditions established for *S. lividans* TK24 (pRL226), the chitosanase production was lower than that in *S. lividans* TK24 (pRL226). Production was particularly low in *S. lividans* TK24 (pFD666 *chs*+); The chitosanase activity was only 0.58 units per ml of fermentation liquid (Table 9, Fig. 8).

We used the SDS-PAGE to analyze the extracelluar proteins produced by the different clones carrying the chitosanase gene. The results showed that the molecular weight of chitosanase was the same (29.5 kDa) for all clones (Fig. 9).

In fact, when the quantitative inoculum less than 1% (mpv) was used, without changing other fermentation conditions, the chitosanase gene in pRL270 could be expressed very well (Fig. 10). Using 0.67% and 4% inocula, chitosanase activity of *S. lividans* TK24 (pRL226) was very similar (about 40 U/ml) , except that the time course of enzyme production was different. But, using 0.67% and 4% of inocula, the chitosanase activity of S. lividans TK24 (pRL270) showed very large difference (47 U/ml and 18 U/ml respectively).

Table 9 Comparison of chitosanase production between the different clones carrying the chitosanase gene

Recombinant Strain: *S. lividans* TK24	Ferm. time* (days)	CHS act. (U/ml)	Specific act. (U/mg-pro.)
1. pRL226	**4**	**74.00**	**66.73**
2. pRL241	4	8.19	25.36
3. pRL228	5	9.94	23.20
4. pRL266	3	19.75	61.15
5. pRL270	4	9.81	28.89
6. pFD666 *chs+*	2	0.58	5.27

* : The time taken for the chitosanase activity to reach the maximum.

** : The ferm. media contained 1.5% Chitosan + 0.5% Starch, as well as 0.2% ammonium sulphate as nitrogen solution.

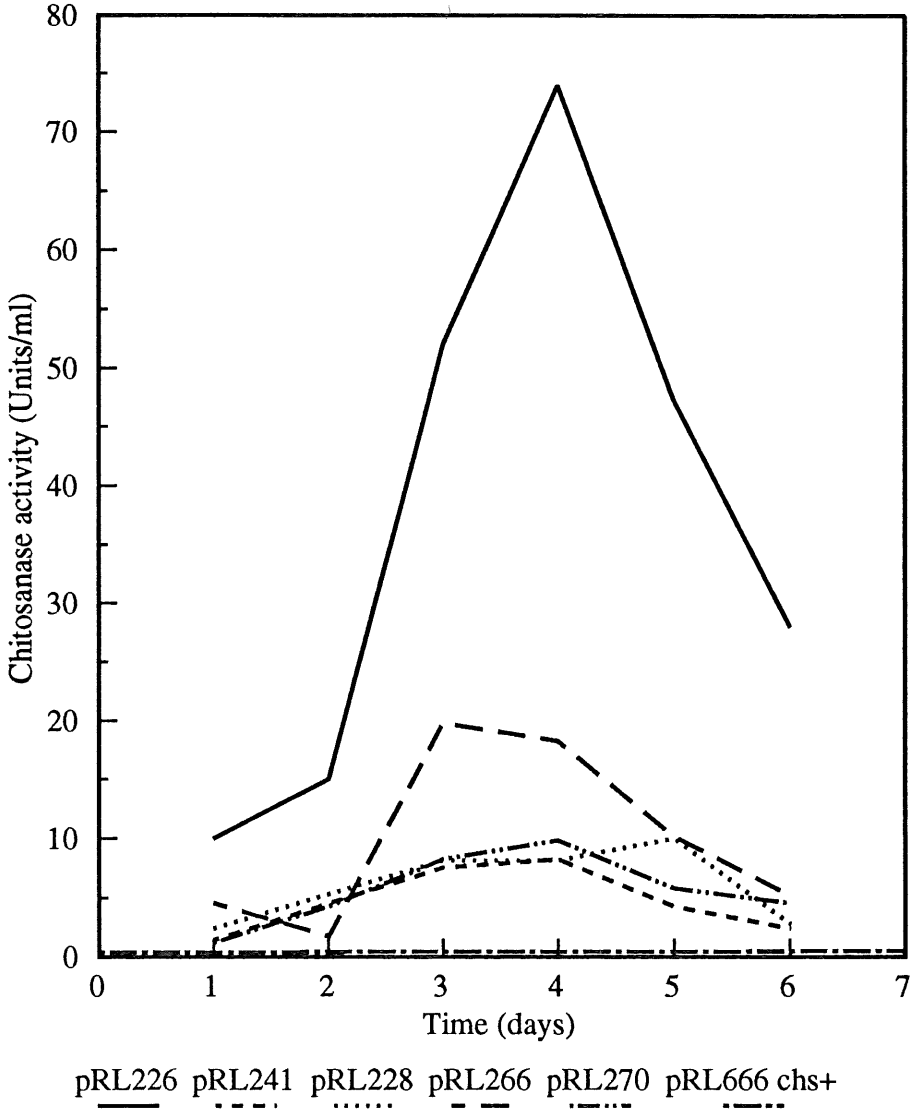


Fig. 8 Comparison of chitosanase production between the different clones carrying the chitosanase gene.

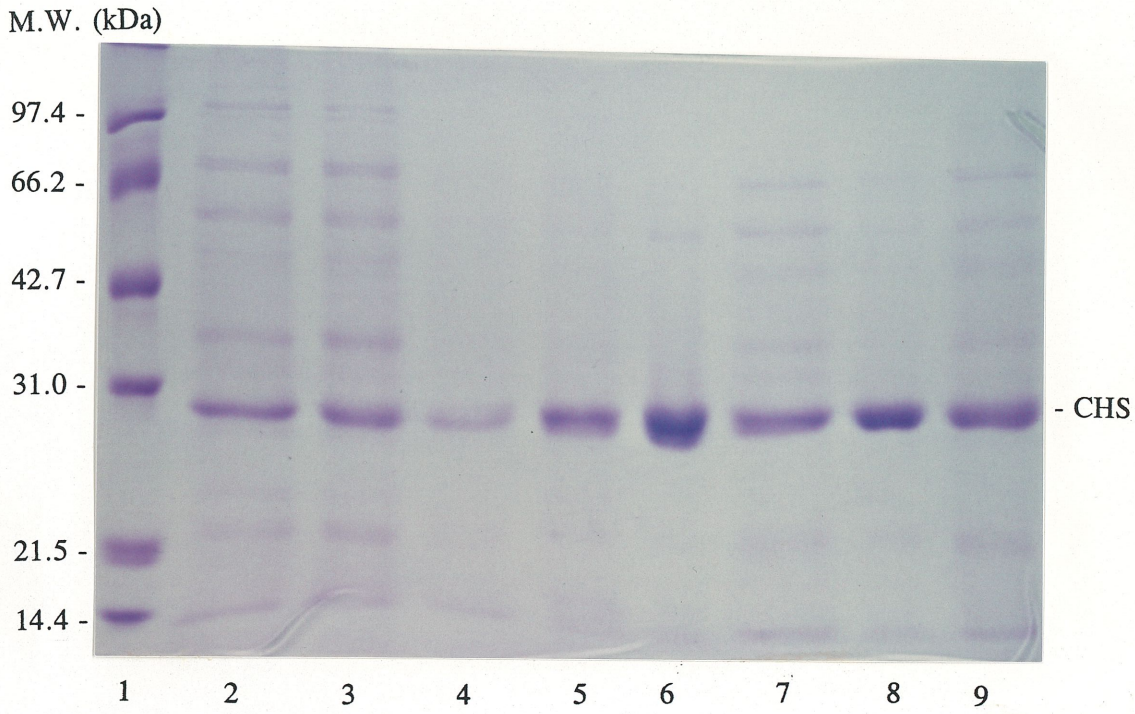


Fig. 9 A SDS-PAGE analysis of crude chitosanase production of different clones in starch and chitosan-starch fermentation media.

1) Standard proteins.

Medium: 2-5) In starch ferm. medium;

6-9) In chitosan-starch ferm. medium.

Strain: 2,9) *S. lividans* TK24 (pRL270); 3,8) *S. lividans* TK24 (pRL266); 4,7) *S. lividans* TK24 (pRL241); 5,6) *S. lividans* TK24 (pRL266).

*) The samples were taken for the chitosanase activity to reach the maximum days.

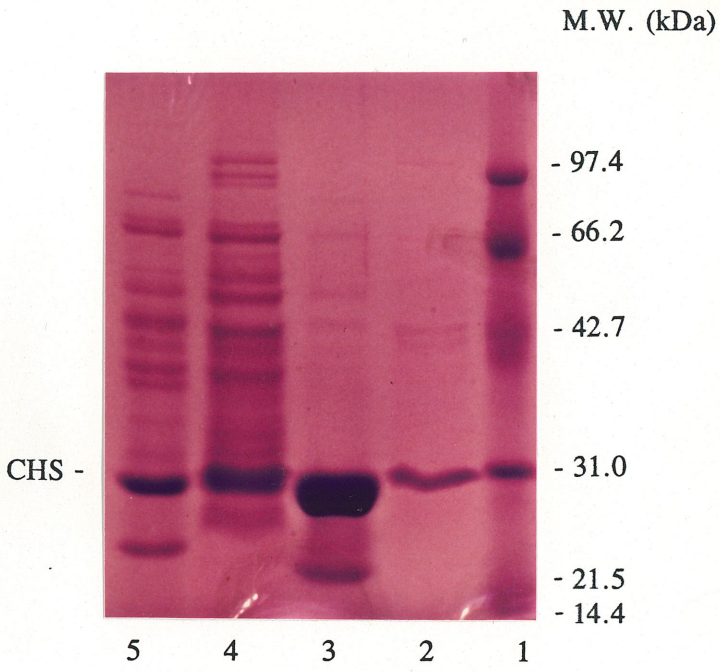


Fig. 9 B SDS-PAGE analysis of crude chitosanase production of different clones in starch and chitosan-starch fermentation media.

1) Standard proteins.

Medium: 2,4) In starch ferm. medium;

3,5) In chitosan-starch ferm. medium.

Strain: 2,3) *S. lividans* TK24 (pRL226); 4,5) *S. lividans* TK24 (pRL228).

*) The samples were taken for the chitosanase activity to reach the maximum days.

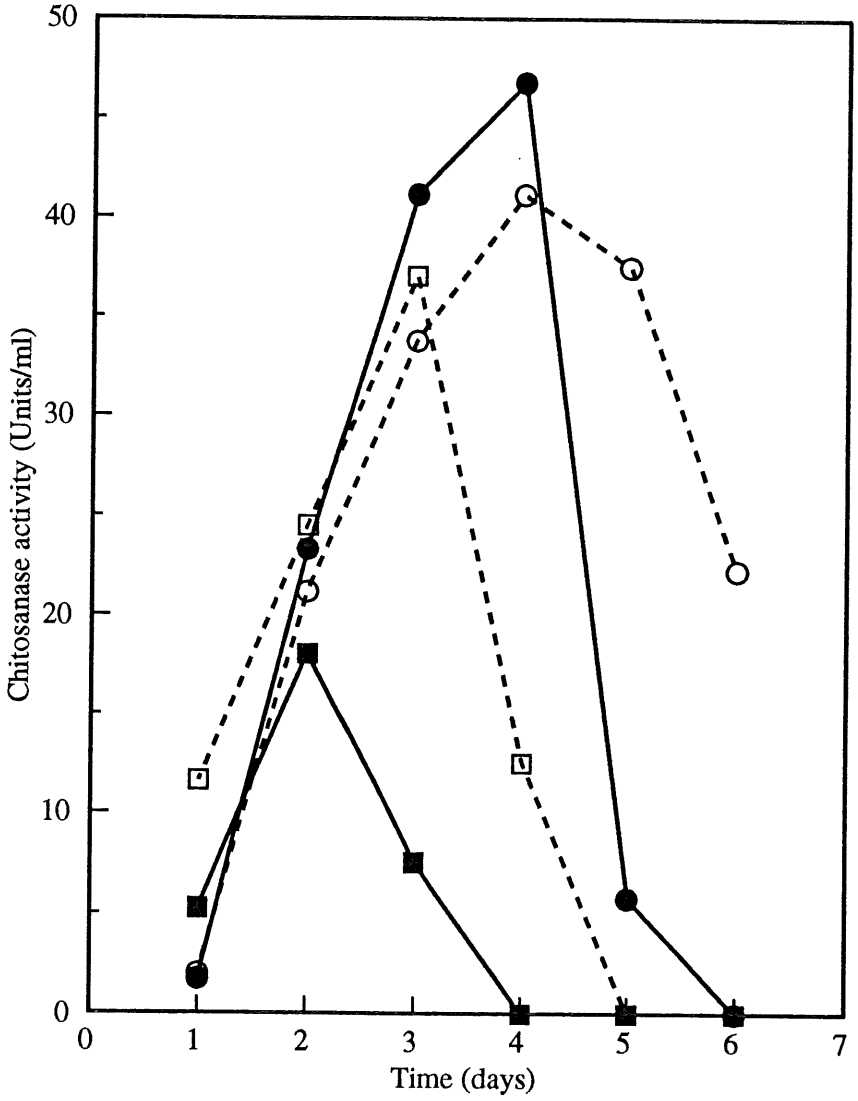


Fig. 10 Comparison of chitosanase production by *S. lividans* TK24 (pRL226) and (pRL270) with different inocula.

○ pRL226 and ● pRL270, inoculated at 0.67 % mpv;

□ pRL226 and ■ pRL270, inoculated at 4.0 % mpv.

3.1.3 Study on the protease in *Streptomyces lividans* TK24

When we studied the chitosanase activity, we found that the time course of chitosanase production was different on various substrates (Fig. 7). For example, the time courses of chitosanase production in starch, chitosan-starch and *M. rouxii* mycelium media, are shown on Fig. 11. In *M. rouxii* mycelium and starch substrates, the chitosanase activity showed slightly decreased after 4 days and returned to the original level after 5 days, remaining stable for a few days. But in chitosan-starch substrates, after 4 days, the activity decreased very quickly and could not be recovered. This probably indicated that concomitant with chitosanase production, a specific proteolytic enzyme for chitosanase was produced in fermentation medium (see section 4.5). To test this hypothesis, we also measured the overall protease activity in fermentation liquid (Fig. 12). We found that there are some relations between the time courses of chitosanase activity and protease activity.

We got similar results in the other experiments. Fig. 13 indicated that in each culture of pRL226 and pRL270, the increase of protease activity precisely corresponded to the moment of a serious decrease of chitosanase activity.

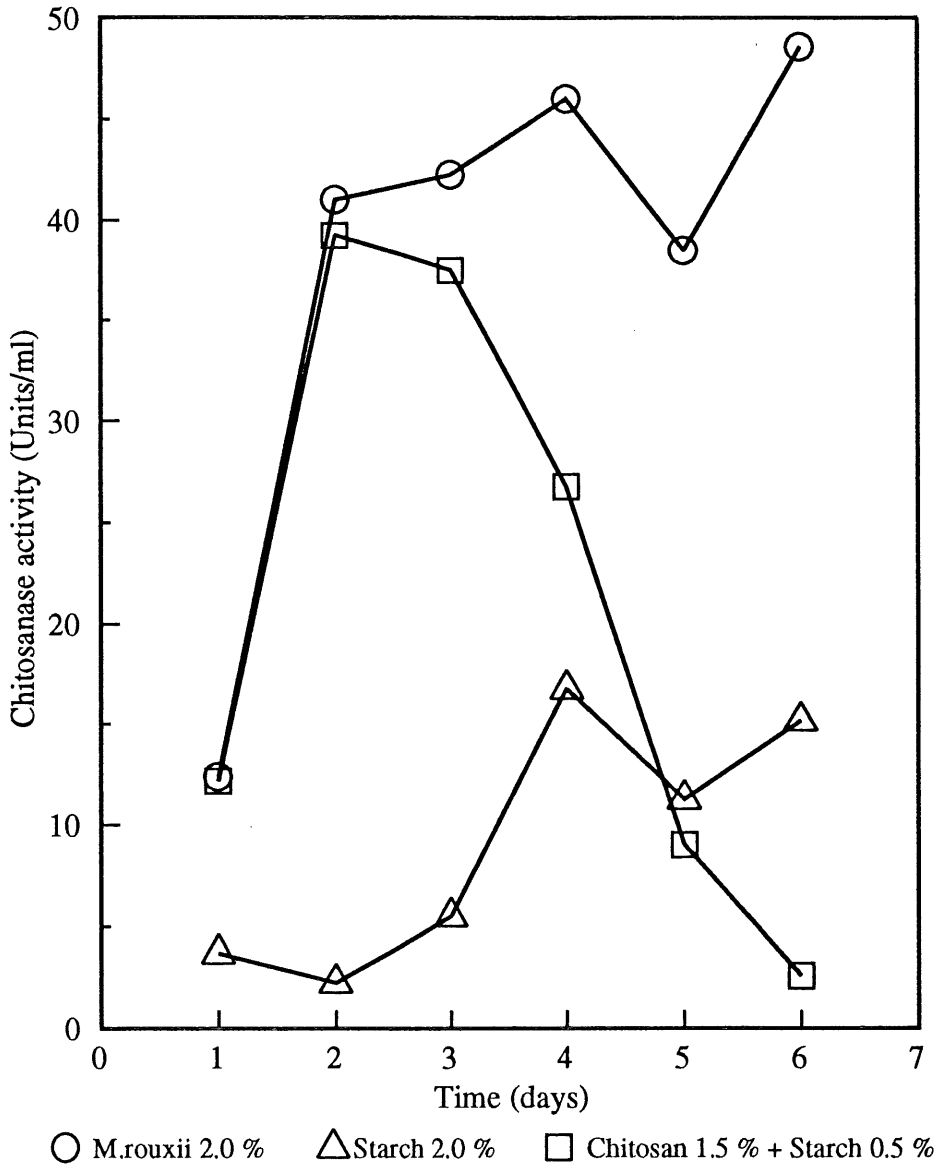


Fig. 11 Time-course of chitosanase production in different fermentation media of *S. lividans* TK24 (pRL226).

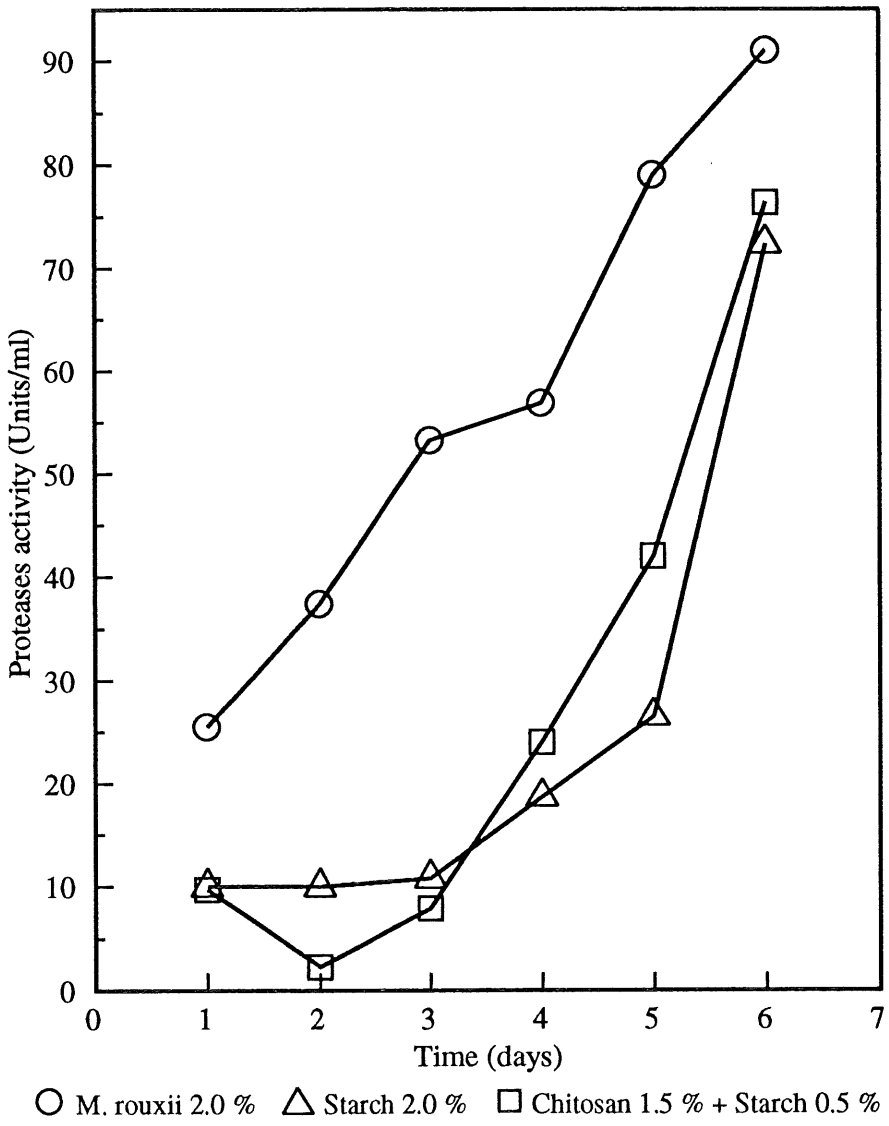


Fig. 12 Time-course of protease activity in different fermentation media of *S. lividans* TK24 (pRL226).

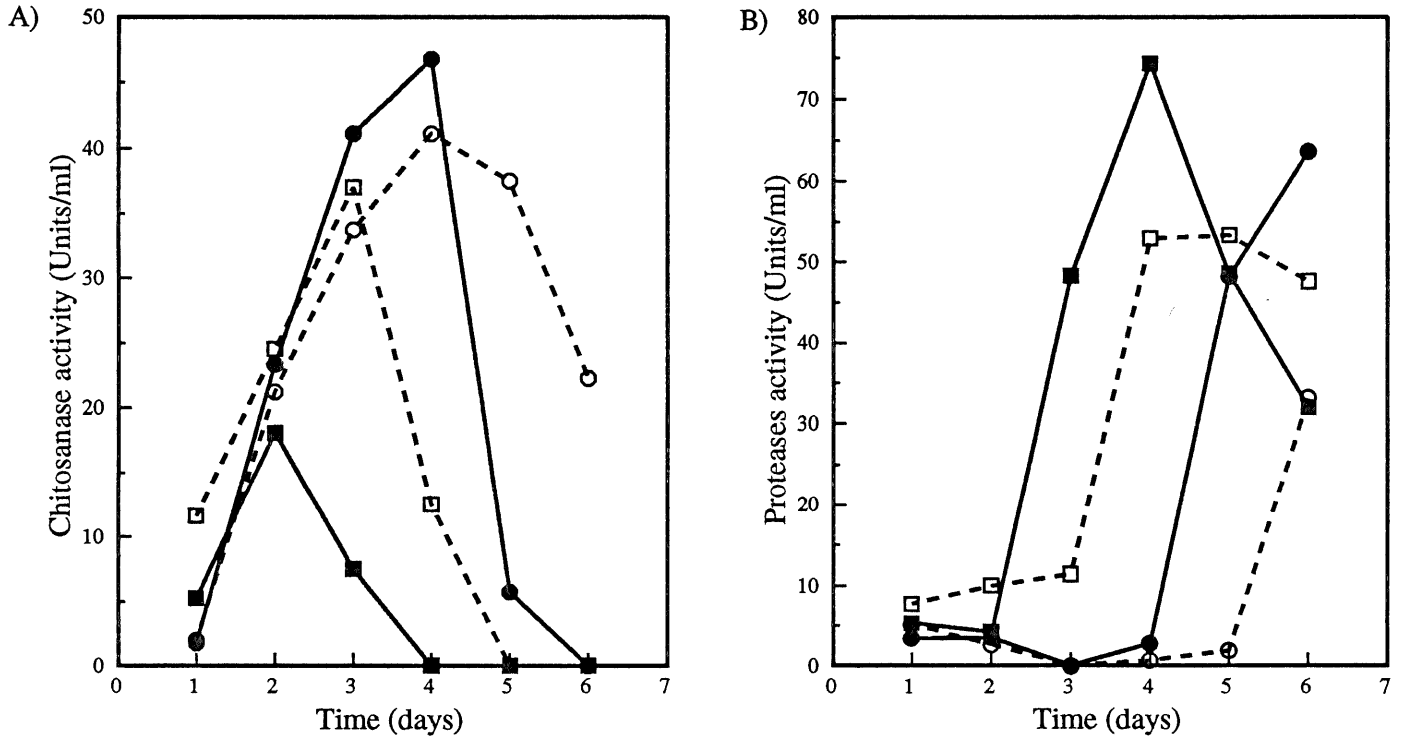


Fig. 13 Time-course of chitosanase production (A) and protease activity (B) in chitosan-starch fermentation media of two *S. lividans* TK24 clones, each inoculated at two different densities.

○ pRL226 and ● pRL270, inoculated at 0.67% mpv;

□ pRL226 and ■ pRL270, inoculated at 4.0 % mpv.

The N174 chitosanase is thus sensitive to degradation by one or several proteases produced by *S. lividans* TK24. This was also illustrated by the progressive decrease of intensity of the chitosanase band observed in Coomasie Blue stained gels (Fig. 14).

The proteolytic degradation of chitosanase activity was not inhibited by PMSF, but it was partially inhibited by 2.5 mM EDTA (data not shown).

For above reasons, we wanted to study chitosanase production in a host strain which is protease deficient or has very low protease activity. A possible candidate for such strain, *S. lividans* 10-164, which has been said to have low protease activity, was offered by Dr. Dieter Kluepfel.

3.1.4. Study on *Streptomyces lividans* 10-164 carrying the chitosanase gene

S. lividans 10-164 is a ß-1,4-glucan-glucanohydrolase (endocellulase) and xylanase-negative double mutant (Mondou et al., 1986). The plasmids pRL226 and pRL270 were transformed into the protoplasts of *S. lividans* 10-164 and two new

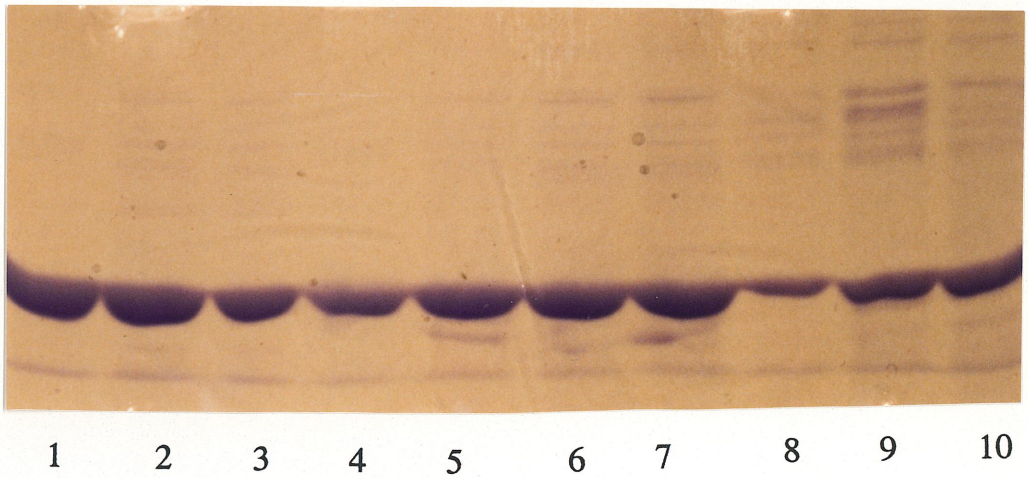


Fig. 14 SDS-PAGE analysis of proteins produced by *S. lividans* TK24 (pRL226) in different fermentation media.

1, 2 and 3: samples were taken from the cultures of 2, 4 and 6 days in 1.5% chitosan + 0.5% M.E. medium;

4, 5, 6 and 7: samples were taken from the cultures of 2, 4, 6 and 8 days in 1.5% *M. rouxii* mycelium + 0.5% M.E. medium;

8, 9 and 10: samples were taken from the cultures of 2, 4 and 6 days in 1.5% *A. niger* mycelium + 0.5% M.E. medium.

recombinant strains were named *S. lividans* 10-164 (pRL226) and *S. lividans* 10-164 (pRL270). The chitosanase gene in these new recombinant strains was expressed very well in glucosamine medium, but expressed at a lower level in chitosan-glucosamine and *M. rouxii* mycelium media (Fig. 15). From these experiments, we found that: (1) the *S. lividans* 10-164 (pRL226) and (pRL270) could use the glucosamine as the carbon and nitrogen source for chitosanase production; (2) the chitosanase activity is stable (after 2 days it reached a high level and this level could be kept for a few days) (Fig. 15); (3) the protease activity of *S. lividans* 10-164 was lower than that in *S. lividans* TK24 (Fig. 16). and (4) no correlation was found between chitosanase and protease activities (Fig. 16).

These suggested that *S. lividans* 10-164 is really different from *S. lividans* TK24 as the host for chitosanase gene expression and that further studies should be necessary to fully explore the potential of this new host.

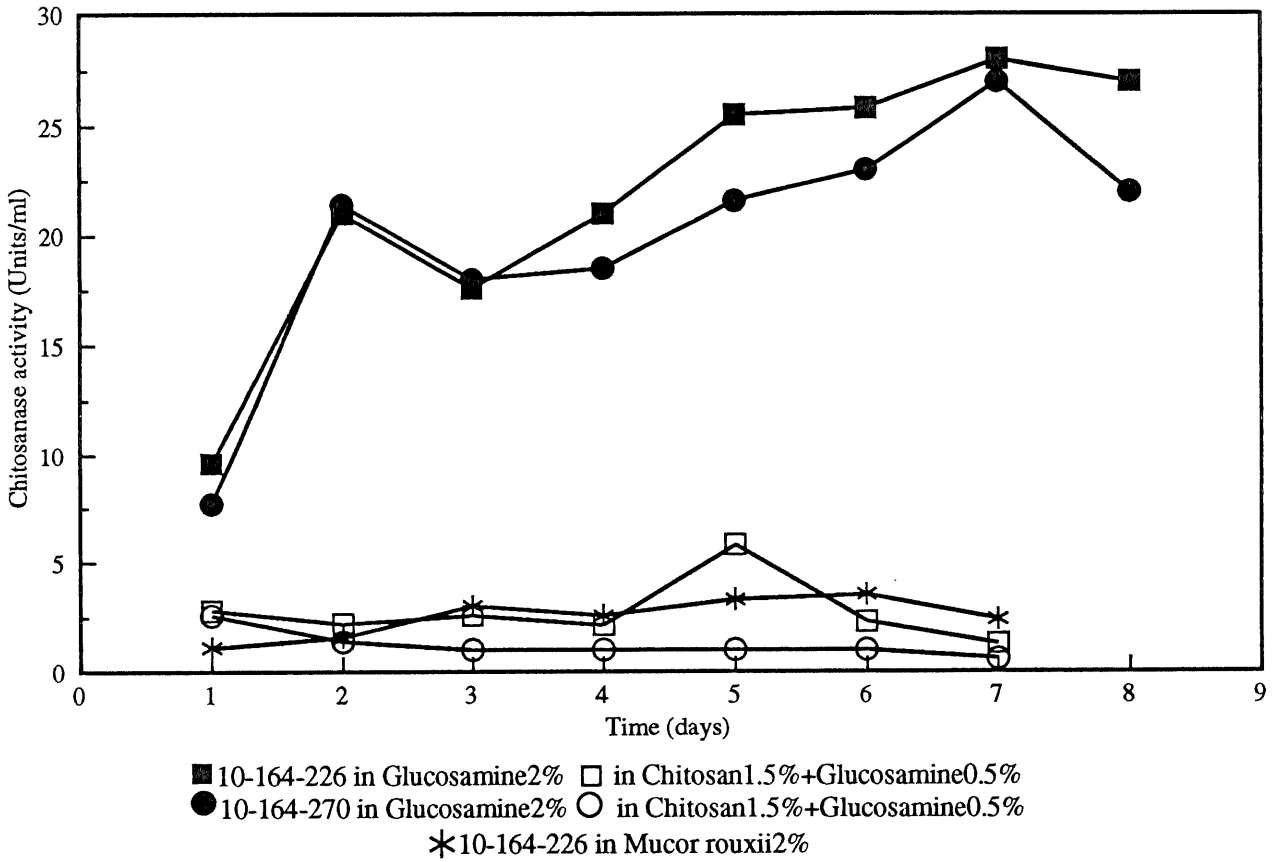


Fig.15 The time course of chitosanase production in fermentation liquid of recombinant strains *S. lividans* 10-164. Inoculum was 1.3 mpv/100ml (MS solution without nitrogen source, the carbon sources are indicated on the figure).

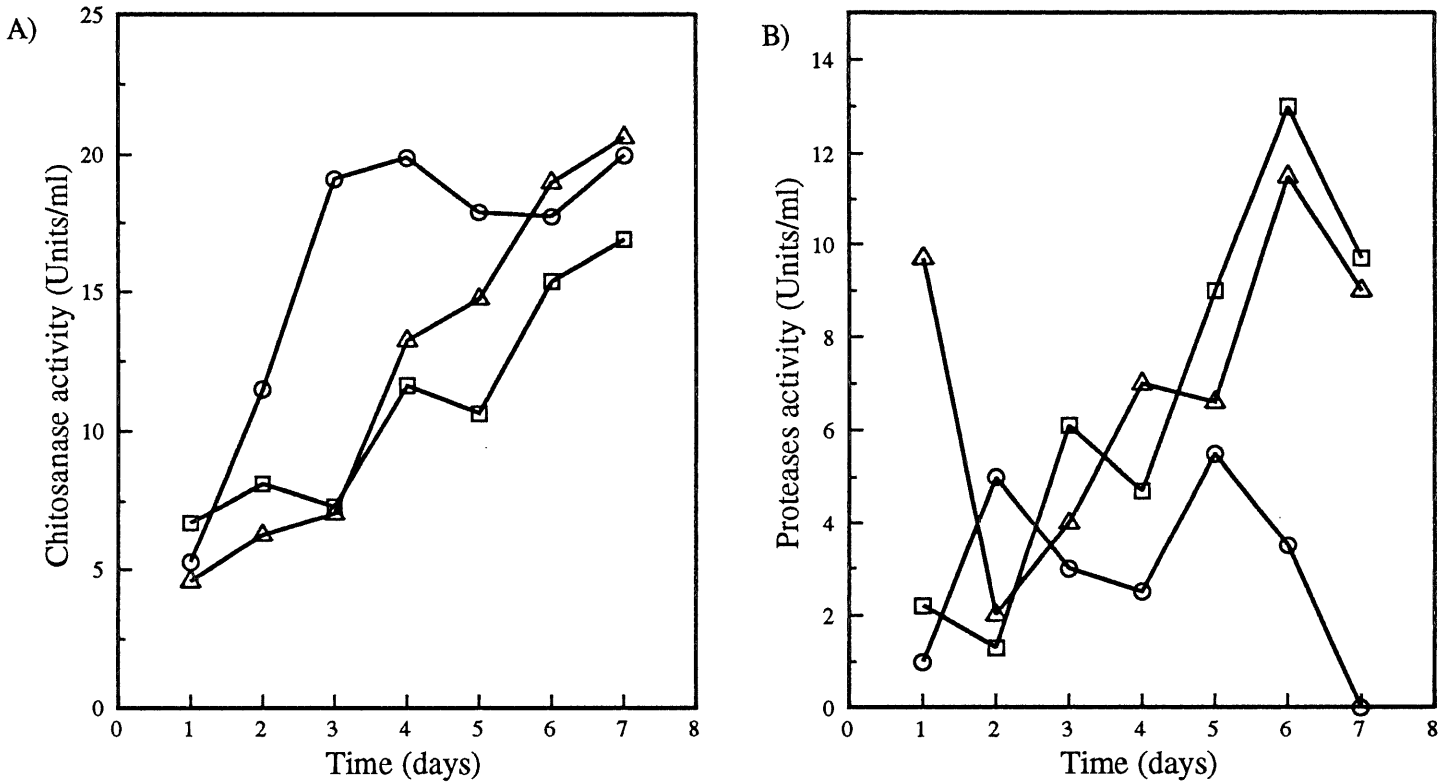


Fig.16 The time course of chitosanase activity (A) and protease activity (B) in fermentation liquid of recombinant strains *S. lividans* 10-164 (pRL226). Inoculum was 1.3 mpv/100ml (MS solution without nitrogen source, the carbon source is indicated on the figure).

○ Glucosamine 1.5% + Starch 0.5% □ Glucosamine 1.5% + M.E. 0.5%

△ Glucosamine 1.5% + Glycerol 0.5%

3.2 Purification of chitosanase with polyacrylic acid

In order to concentrate and stabilize the chitosanase from the fermentation liquid in an economical way, we precipitated the chitosanase protein with PA at pH 4 (Table 10). This crude enzyme extract could be stored for one year at -20°C in 50% glycerol, retaining 50% of its activity. After precipitation, the overall proteolytic activity decreased to 5% of the initial level. This suggested that most of the proteolytic activity produced by *S. lividans* TK24 have acidic pI's. Furthermore, the activity that degrades the chitosanase protein became undetectable when the PA precipitate was dissolved in a buffer containing 2.5 mM EDTA (data not shown).

3.3 Preparation of the active chitosan oligomers by chitosanase hydrolysis

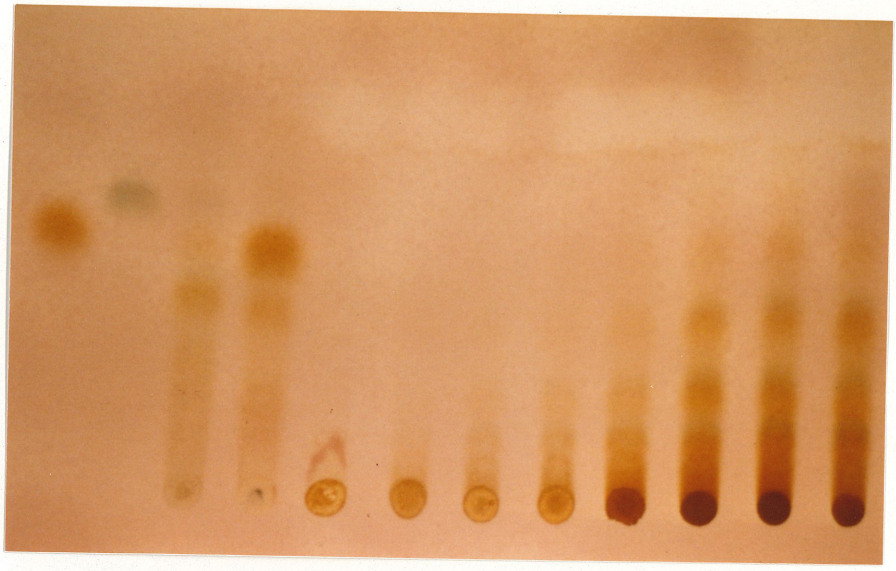
In order to find the optimal degree of hydrolysis of chitosan, chitosan solutions were incubated with different concentrations of chitosanase. The products of hydrolysis of chitosan by the chitosanase were analyzed by thin layer

Table 10 Purification of chitosanase with polyacrylic acid.

	Ferm. liquid	Precipitation of enzyme
Volume (ml)	160	45
Proteins (mg/ml)	1.88	3.56
Activity (U/ml)	80	160
Total protein (mg)	301	160
Total act. (units)	12800	7200
Specific act. (U/mg)	42	45
Yield (%)	100	57
Purification factor	1	1.1

chromatography. The results, shown in Fig. 17, indicated that the chitosanase first hydrolysed chitosan to produce longer chitooligosaccharides and then these oligosaccharides were hydrolysed to shorter chitooligosaccharides as the final products. This mechanism is similar to that described for the chitosanase from *Nocardia orientalis* (Sakai et al., 1991)

The results clearly showed that the enzyme hydrolysed chitosan to produce long chitooligosaccharides, such as chitopentaose, chitohexaose, chitoheptaose and longer oligomers, when 1 to 2 units of chitosanase were added to chitosanase solution (Fig. 18). According to Kendra and Hadwiger (1984), we should select one unit chitosanase per 250 mg of chitosan as the optimal concentration for preparation of the active chitosan oligomers, which should display maximal activities in both fungal growth inhibition and induction of defense responses in plants.



1 2 3 4 5 6 7 8 9 10 11 12

Fig. 17 Thin layer chromatography showing the different degrees of chitosan hydrolysis by crude chitosanase.

1) Standard of GlcN; 2) Standard of GlcNAc;

3,4) Oligomers of GlcN;

5-12) 250 mg of chitosan was hydrolysed by different units of chitosanase (0.5U, 1U, 1.5U, 2U, 3U, 4U, 5U and 6U). (see section 2.6.1).

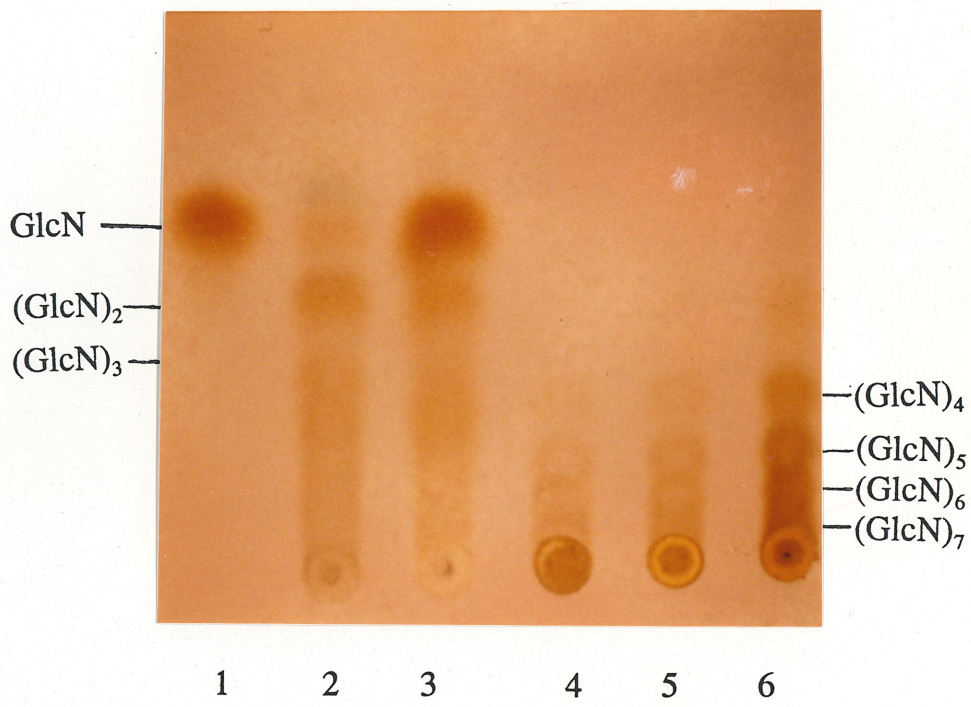


Fig. 18 Thin layer chromatography showing the optimal degree of chitosan hydrolysis by crude chitosanase.

1) Standard of GlcN;

2,3) Oligomers of GlcN;

4) 250 mg of chitosan hydrolysed with 1U of CHS.

5) 250 mg of chitosan hydrolysed with 1.5U of CHS.

6) 250 mg of chitosan hydrolysed with 2U of CHS.

3.4 Antifungal properties of the chitosan oligomers and induction of defensive responses in plants

After getting the high-molecular-weight chitosan oligomers, we pursued the possibility of their commercial use. We analysed their antifungal properties and their ability to induce defense reactions in plants.

3.4.1 Inhibition of fungal growth

Fusarium oxysporum f. sp. *radicis-lycopersici* is a widespread soil borne pathogen that is responsible for severe tomato yield losses in green house soil and hydroponic systems (Jarvis, 1989; Jenkins and Averre, 1983). As with other vascular wilt pathogens (Beckman, 1987), *F. oxysporum* f. sp. *radicis-lycopersici* gains entry into roots through wounds and colonizes in the direction of xylem vessels, where it abundantly multiplies (Charest et al., 1984).

Our results showed that the chitosan oligomers prepared by our procedure significantly reduced the radial growth of *F. oxysporum* f.sp. *radicis-lycopersici* (Table 11). The marked effect was observed at a concentration of 2 mg/ml, which

indicated a growth inhibition of 60%. The fungal hyphae growth on plates containing chitosan oligomers was dense and short, whereas the fungal growth on control plates developed normally and actively (Fig. 19).

Table 11 Effect of chitosan oligomers on inhibition of radial growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici*.

PDA containing different concentration of chitosan oligomers	The diameter of radial growth of 5-day-old *F.oxysporum* (mm)[a]	Inhibition of radial growth (%)
0.00 mg/ml	72(±1.1)[b]	0
0.25 mg/ml	67(±2.6)	7.6
0.50 mg/ml	60(±1.0)	18.2
1.00 mg/ml	51(±1.2)	31.8
2.00 mg/ml	32(±1.5)	60.6

[a]. Fungi were inoculated with a 6-mm-diameter mycelial plug taken from the margin a 5-day-old *Fusarium oxysporum f.*sp.*radiacis-lycopersici* culture.

[b]. Values in parentheses are standard errors of mean.

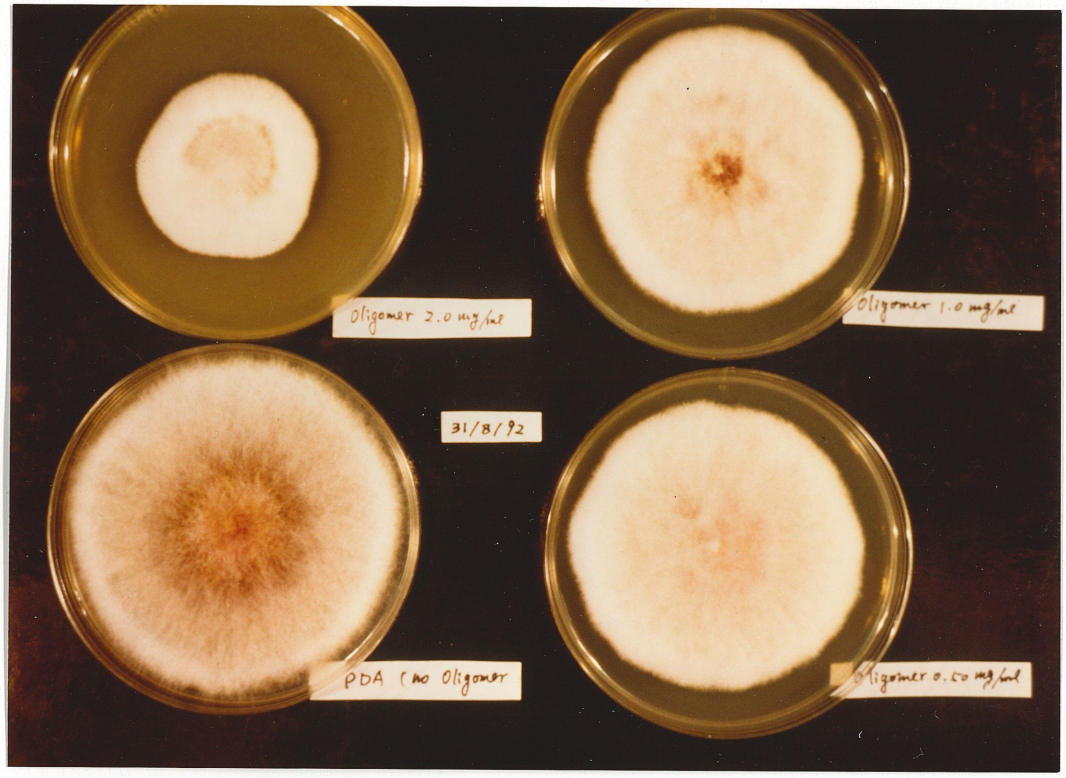


Fig. 19 Effect of chitosan oligomers on inhibition of radial growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici*. (The PDA medium contains chitosan oligomers with different concentrations shown in figure).

3.4.2. Induction of pathogenesis-related proteins during tomato seeds germination process

Our attention was focused on the biological function of chitosan oligomers present during seeds germination process for increasing the plant resistance. Our experiment results showed that P.-R. proteins were induced by our chitosan oligomers during tomato seeds germination process.

Chitinase, ß-1,3,-glucanase and chitosanase were induced during tomato seeds germination process by the chitosan oligomers, and the enzymatic activities were measured in 6-day-old tomato seedlings. The activities increased along with the increase of concentration of chitosan oligomers (Fig. 20-22), but a toxic effect for tomato seedlings was observed when the chitosan oligomers were used at a concentration higher than 500 μg/ml. This phenomenon was also described with bean plants (Pospieszny and Atabekov, 1989). The optimal concentration of the chitosan oligomers for induction of P.-R. proteins synthesis was 200 μg/ml. In addition, Table 12, which shows the effect of chitosan oligomers on tomato seeds germination efficiency, also indicated that our chitosan oligomers were of benefit to tomato seeds germination at a proper concentration (lower than 200 μg/ml).

Higher concentrations (such as 500 μg/ml) were toxic and inhibited germination.

Table 12 Effect of chitosan oligomers on tomato seeds germination.

Concentration of chitosan oligomers in 0.6% agar plates	**Germination (%) (After incubation days)**					
	1[a]	**2**	**3**	**4**	**5**	**6**
water	0	56[b]	84	85	85	85[c]
50μg/ml	0	52	85	88	89	89
100μg/ml	0	47	90	90	90	90
200μg/ml	0	41	80	88	90	90
500μg/ml	0	0	68	78	80	80
1000μg/ml	0	0	47	68	77	80

[a]. The number is the day of incubation.

[b]. Each seed germination experiment was done with 100 seeds.

[c]. The producer also expected 85% germination of those seeds.

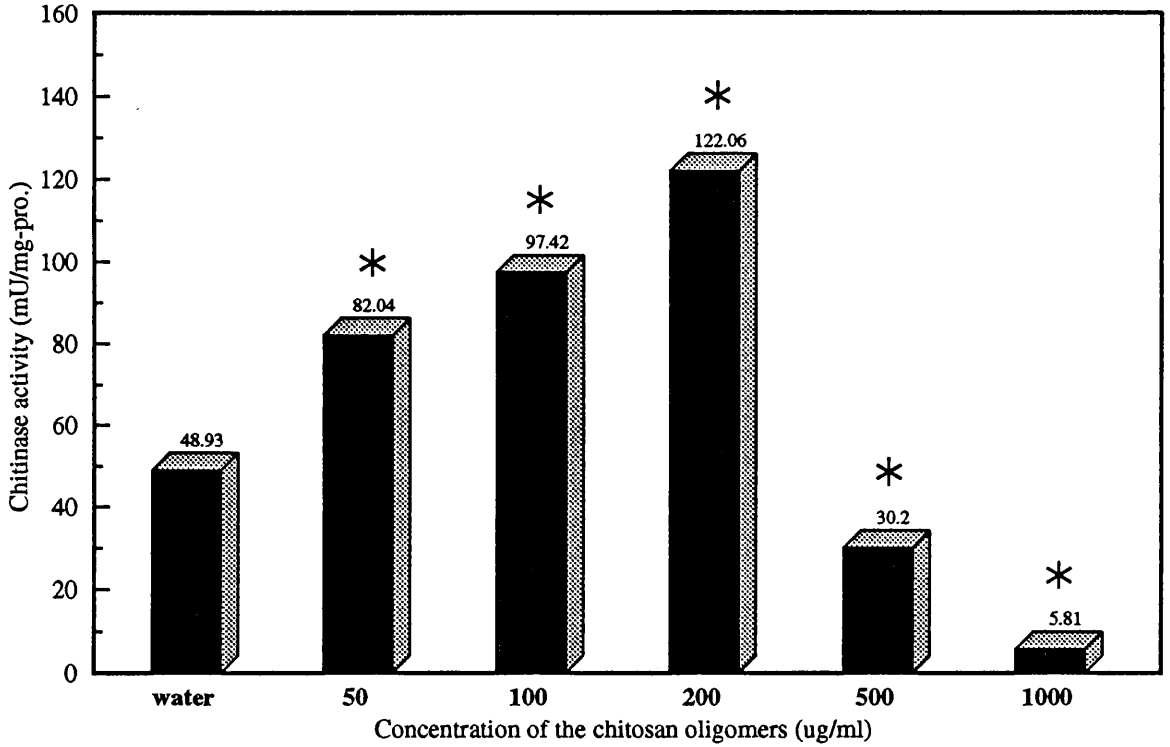


Fig. 20 Induction of chitinase activity by the chitosan oligomers in tomato seedlings.

*: The datum has significant difference with control.

**: Each of the data is expressed per 100 seedlings and is an average of three experiments.

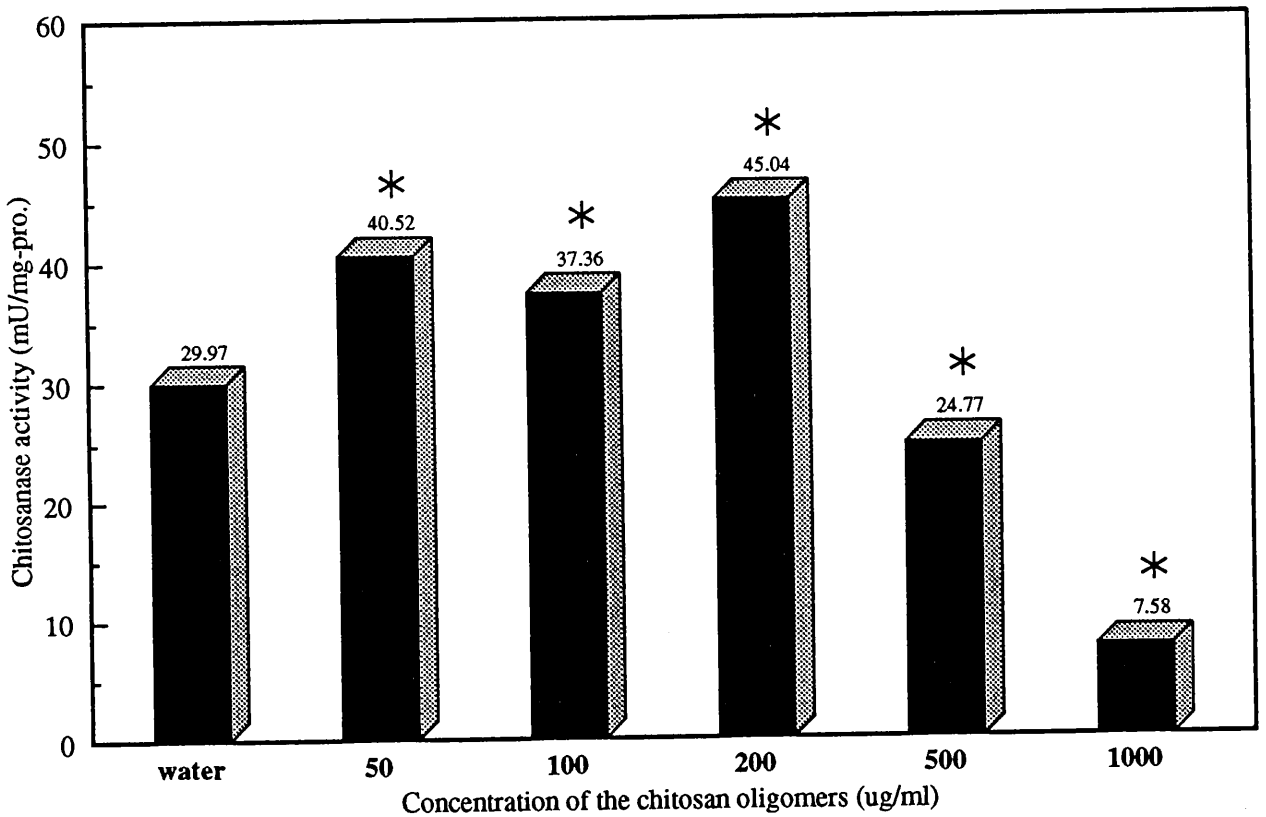


Fig. 21 Induction of chitosanase activity by the chitosan oligomers in tomato seedlings.

*: The datum has significant difference with control.

**: Each of the data is expressed per 100 seedlings and is an average of three experiments.

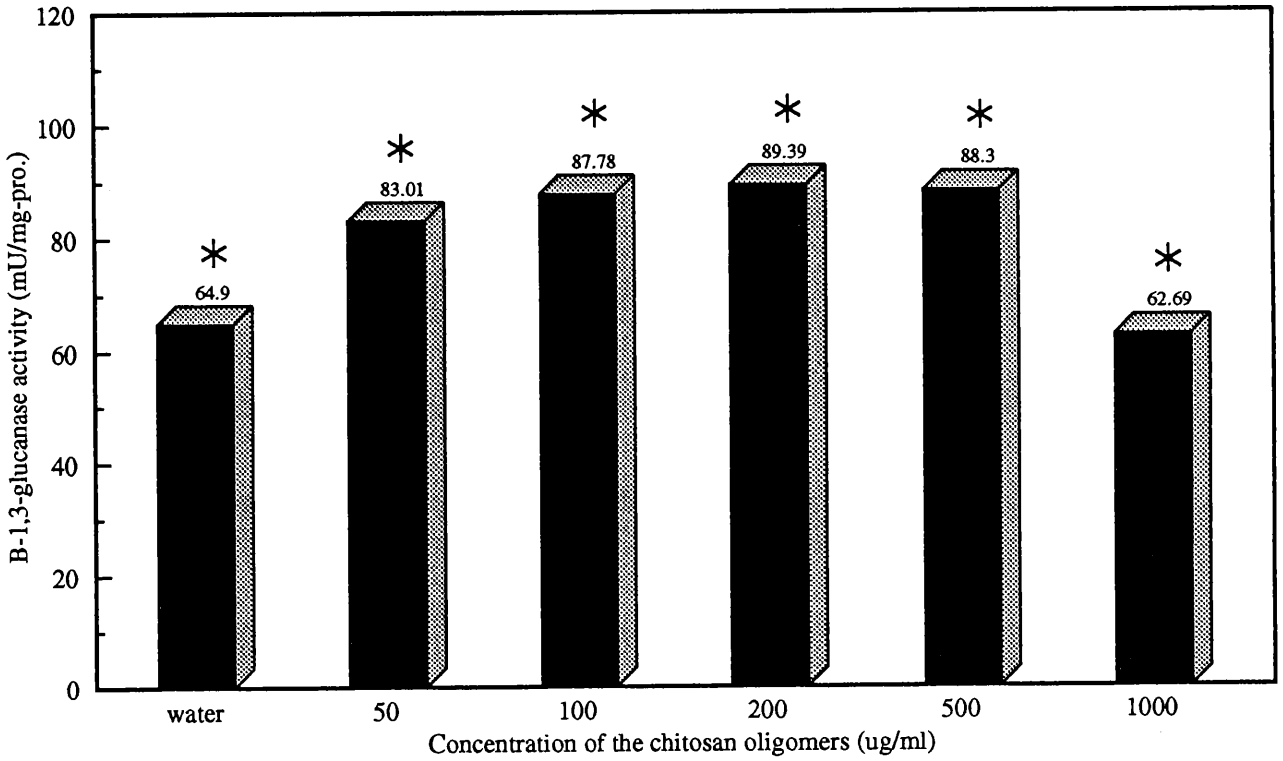


Fig. 22 Induction of ß-1,3-glucanase activity by the chitosan oligomers in tomato seedlings.

*: The datum has significant difference with control.

**: Each of the data is expressed per 100 seedlings and is an average of three experiments.

3.4.3 Induction of pathogenesis-related proteins in mature plants

In order to test the effect of our chitosan oligomers on the induction of P.-R. proteins synthesis in mature plants, we sprayed the chitosan oligomers on a mature plant leaf. After waiting for the induction of proteins synthesis and systemic movement from treated to untreated leaves (about 5 days), three plant leaves were collected from each plant (one from plant upper part, one from middle part, another from lower part). The intercellular fluid of leaves was collected and the enzymatic activities of P.-R. proteins were measured (Table 13-14).

The experiment showed that the P.-R. proteins were induced by the chitosan oligomers in tobacco leaves. The enzymatic activities of chitosanase, chitinase and ß-1,3-glucanase of plant fluids obtained from tobacco leaves treated with the chitosan oligomers at the concentration of 10 mg/ml were significantly different from the control plants (Table 13). We repeated this experiment three times and got similar results. These indicated that the chitosan oligomers were efficient to induce the synthesis of P.-R. proteins in plants.

Table 13 Enzymatic activities in tobacco leaves treated with chitosan oligomers.

Concentration of the chitosan oligomers	Chitosanase activity (U/mg-pro.)	Chitinase activity (U/mg-pro.)	β-1,3-glucanase activity (U/mg-pro.)
Water	0.468	0.810	0.251
1mg/ml	0.492	0.917	0.287
10mg/ml	0.827*	1.349*	0.420*

*: The value has the significant difference with control.

**: All of the data are the average of three plants.

Table 14 Enzymatic activities in tomato leaves treated with chitosan oligomers.

Concentration of the chitosan oligomers	Chitosanase activity (U/mg-pro.)	Chitinase activity (U/mg-pro.)	β-1,3-glucanase activity (U/mg-pro.)
Water	1.33	1.83	0.59
1 mg/ml	1.77*	2.37*	0.52
10 mg/ml	1.44	2.09	0.75*

*: The value has the significant difference with control.

**: All of the data are the average of three plants.

We also did the same experiment with tomato plants. The results of the first experiment is shown in Table 14. Chitosanase and chitinase activities could be induced by the chitosan oligomers at the concentration of 1 mg/ml; at a concentration of 10 mg/ml, the chitosanase and chitinase activities were not significantly different from the control plants. The activity of ß-1,3-glucanase could be induced only by the chitosan oligomers at the concentration of 10 mg/ml. We repeated this experiment but we did not get similar results (see section 4.6.1). Some further studies should be done in the future.

CHAPTER 4

DISCUSSION

4.1 *Streptomyces*: a good host for heterologous gene expression

The most commonly used prokaryotic host for the production of heterologous proteins is the gram-negative bacterium *Escherichia coli* Although high levels of expression can be obtained from this bacterium, heterologous proteins are generally retained within the cells as an insoluble and often biologically inactive inclusion bodies (Wang, 1988). As a result of these problems, alternative hosts capable of protein excretion have been investigated.

An alternative, protein-excreting prokaryotic host which is recently gaining attention is *Streptomyces* (Crawford, 1988). Gram-positive bacteria differ from *E. coli* in that they have a single outer membrane; consequently proteins secreted from *Streptomyces* are deposited directly into the culture medium. The advantages in producing proteins in their secreted form are significant. It allows easy recovery of the enzymes and separation from all intracellular proteins, often in the correctly folded structure and free of the N-terminal methionine, which is of particular

importance for peptides used as therapeutic agents. This prokaryote is commonly used in the pharmaceutical industry, and appropriate vectors have been developed to permit genetic modifications. However, little work was done so far to understand the rules governing the production of heterologous proteins by *Streptomyces*.

In our laboratory, a series of subclones carrying the chitosanase gene from *Streptomyces* N174 and some flanking sequences was constructed in pFD666, a shuttle vector of *E. coli* and *Streptomyces*. Chitosanase production was studied in both hosts. The level of expression in *E. coli* was very low (lower than 0.01 units per ml of fermentation liquid) and the chitosanase gene was transcribed mainly from a vector promoter. The processing of the signal peptide was heterogenous: up to three different chitosanase forms were detected among the periplasmic proteins by Western blotting. Analysis of the signal peptide sequence showed possible alternative cleavage sites (Masson et al., 1992). In contrast, *S. lividans* produced chitosanase very efficiently and only one enzyme form was secreted into fermentation medium (Fig. 9). In optimal conditions, the chitosanase activity reached up to 95 units per ml of fermentation liquid. Until now, this was the best result for chitosanase production. Our experiments is a good example of use

Streptomyces strains as a host for successful expression of a foreign gene.

Although it is clear that *Streptomyces* which is far behind *E. coli* in terms of the number of sophisticated tools available for the manipulation of gene expression, offers important potential advantages over *E. coli* for the expression and secretion of some proteins.

4.2 Pre-culture to help *Streptomyces* spore germination

In preliminary experiment, we found that a pre-culture of *S. lividans* in TSB medium to help spore germination is necessary. The onset of protein synthesis takes place during early germination of *Streptomyces* spores. It is assumed that dormant spores have a less efficient protein biosynthesis machinery. This "defect" is localized on ribosomes and hence, during the first minute of spore germination, the ribosomes must be reactivated for germination tube emergence and primary vegetative mycelium elongation (Vanek et al., 1988).

According to Vanek et al. (1988), the quality and quantity of both the primary and secondary metabolism are determined during the germination of

Streptomyces spore. In average conditions primary vegetative metabolism in complex medium is terminated after 18-24 hours, and protease activity was maximal during mid-exponential growth (Shapiro, 1989). Consequently, we should control the pre-culture about 20-24 hours, and be sure that the pre-culture grows in the early exponential phase. It is very important to transfer the pre-culture into the optimal fermentation medium in good time for chitosanase gene expression.

4.3 Investigation on possible induction of chitosanase gene expression by chitosan

To test whether the chitosanase gene expression could be regulated by induction of chitosan, two subclones (pRL 226X and pRL 270X) were constructed in which the chitosan coding sequence was replaced by the *xylE* promoterless reporter gene (Masson et al., 1993). These plasmids were transformed into *S. lividans* TK24 and the *xylE* gene transcriptional activities in the absence or in the presence of chitosan (which was partially predigested to maximize eventual induction) were measured. The results did not confirm the induction hypothesis. *xylE* activities in starch medium and in chitosan-starch medium were similar among clones. But chitosanase production was clearly higher in the presence of

chitosan than in the absence of chitosan (Table 4). It is however possible that the physical presence of the chitosanase protein (or gene) is necessary for induction and that could explain why the reporter gene expression was not induced on plasmids pRL226X and pRL270X.

According to Bartnicki-Garcia (1973), *M. rouxii* fungal cell walls contain 32.7% GlcN. Reyes et al. (1985) found that *M. rouxii* mycelium could probably be a good natural inducer of chitosanase during the *M. rouxii* production of autolytic chitosanase. Consequently to the action of chitosanase on *M. rouxii* cell wall elements, low levels of glucosamine and oligosaccharides are formed as result of the hydrolysis of chitosan and these compounds can induce chitosanase production in *S. lividans*.

4.4 Function of other DNA sequences in the cloned chitosanase gene

In the same fermentation conditions, expression of the chitosanase gene in recombinant strain *S. lividans* TK24 (pFD666 *chs*+) was about 20 times lower than in *S. lividans* TK24 (pRL270) (Fig. 3, Fig. 8 and Table 9). However, the

difference between pFD666 *chs*+ and pRL270 is just in the promoter upstream the chitosanase gene coding sequences. This result puzzled us. According to Masson et al. (1993) the chitosanase gene transcription should be constitutive and driven mainly from the vector promoter (the synthetic promoter).

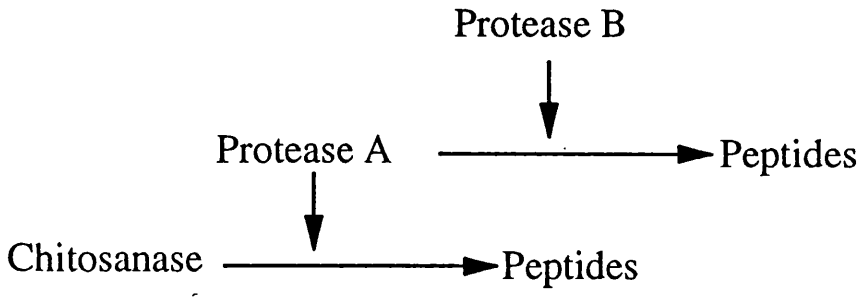
Until now, we know that the sequence after the chitosanase gene has some function on mRNA stability (Brzezinski, R. and Masson, J.-Y. 1993, personal communication). But there is a need to study more deeply the function of the DNA sequences upstream from the chitosanase gene. These sequences should have some functions for chitosanase gene expression. Because, in the same fermentation conditions, the strain carrying the pRL266 plasmid produced chitosanase activity twice more than pRL270 carrying strain and the specific activity was very high (61.15 U/mg-pro.) in *S. lividans* TK24 (pRL226) fermentation conditions (Table 9). Another possibility, however, is that every clone needs a separate optimization of the fermentation conditions and thus that the conditions established for *S. lividans* TK24 (pRL226) are far from optimal for the other clones.

The results of Fig. 10 and those obtained for pRL226X and pRL270X subclones, (Masson et al., 1993) also suggested that a separate optimization of the

inoculum should be made for every clone studied. Maybe this is why the recombinant strains carrying pRL241, pRL228, pRL266 and pFD666 *chs*+ showed a lower expression of the chitosanase gene than that of pRL226 in optimal fermentation condition of pRL226.

4.5 Extracellular protease in *Streptomyces lividans* TK24

A possible hypothesis explaining why chitosanase production time courses are so different on various substrates (Fig. 11) is that a specific proteolytic enzyme for chitosanase was concomitantly produced in fermentation medium. From Fig. 12 and Fig. 13, we knew that there were some relations between the time course of chitosanase activity and protease activity. We summed up these results and suggested the following model to explain them.



The hypothetic protease A could degrade chitosanase into inactive peptides. However, these protease would be presented only transiently in some fermentation media, because it would be degraded by the protease B which is produced later in these media.

In the culture supernatants of media with starch or *M. rouxii* mycelium as carbon source the specific protease A for hydrolysis chitosanase was produced by *S. lividans* TK24 (pRL226) after 4 days, and the hypothetic protease B (hydrolytic for protease A) would be produced after 5 days. In fact, the chitosanase activity decreased a little at 4 or 5 days, but it can be recovered after 5 days, because the protease B stopped the activity of protease A. However, in the chitosan-starch fermentation medium, the specific protease A was produced after 2 days (during 2nd and 3rd day the protease activity increased slowly, the chitosanase activity decreased slowly; the proteases activity increased fast after 3 days; the chitosanase activity decreased fast). No protease B was produced afterward or the protease B would appear very later (Fig. 11 and Fig. 12). Consequently, the chitosanase activity could not be recovered in later stages of culture. We got similar results with *S. lividans* TK24 (pRL270) at a various amounts of inocula, as shown in Fig. 13. We found that protease levels were low at early culture stages but increased

quickly at the onset of the stationary phase. The protease A and protease B are post-growth associated activities. Similar results were reported with other *S. lividans* strains (Aretz et al., 1989, Aphale and Strohl, 1993). At least a part of the "protease A" is metalloprotease, because the protease A could be inhibited partially by EDTA.

Protein stability is another factor that significantly influences the ability to achieve stable expression of foreign gene products. Optimization of growth conditions by prolonging active growth or inclusion of nutrients that limit protease activity and expression are strategies that might be used to suppress protease expression. Very little is known about the effects of nutrient availability and depletion on protease expression until now. Although proteolysis has not significantly limited the production of certain proteins, it is likely that productivity could be enhanced by limiting proteolysis at specific stages during the growth cycle (Brawner et al.,1991).

4.6 Antifungal properties of chitosan oligomers and induction of defensive responses in plants

4.6.1 Solution of chitosan oligomers

Chitosan and high-molecular-weight chitosan oligomers can not be dissolved in water, they can be just dissolved in an acid (e.g. acetic acid). If the solution is neutralized by sodium acetate, the solution will contain acetic acid and Na^+. Until now, most of published work used acidic solution of chitosan for testing antifungal properties and activity to induce defense responses in plants. Of course, few works used acid solution without chitosan as a control (Pospieszny and Atabekov, 1989). But, according to our experimental results, inhibition of fungal growth in control experiments using acid solution without chitosan oligomers is more powerful than that of the same acid solution with chitosan oligomers, even if their pH are at the same level (pH 5.5). We repeated this experiment three times, the results were similar. We can not explain it until now. After eliminating HAc by ethanol and ether (see section 2.6.1), the purified high-molecular-weight chitosan oligomers were obtained, but could not be dissolved in water. We could suspend them as particles in water. Using this suspension to treat plants, we had to raise the

concentration of chitosan oligomers because it is difficult to absorb the particles of chitosan oligomers on plant leaves. Probably, that is why the repeated experiments on mature tomato were not successful (see section 3.4.3).

4.6.2 Interaction of chitosan oligomers, fungus and plants

The genus *Fusarium* includes a number of plant pathogens, and it was reported that chitosan was a component of the cell walls of the *Fusarium* strains (Hadwiger and Beckman, 1980). In the interaction between pea pod tissue and *F. solani* f. sp. *phaseoli*, the chitosan oligomers were released from the fungal cell wall. These chitosan oligomers were also supposed to serve as an inhibitor for fungal attack, since chitosan released from fungal cell wall was found to be a potent growth inhibitor for the fungal strain itself (Hadwiger et al., 1981; Kendra et al., 1989).

Recently, Shimosaka et al. (1993) reported that the secretion of significant chitosanolytic activity was found in various strains of the genus *Fusarium*. There is a possibility that the chitosanase is involved in the autolysis of this fungus by degrading the cell walls of the aged mycelium as reported in *M. rouxii* (Reyes et

al., 1985). It remains to be examined whether the chitosanase is secreted by the fungi in the process of fungi-plant host interaction. And if secreted, does the enzyme degrade the fungal cell walls and release chitosan or chitosan oligomers acting as an elicitor? Elucidation of a role of the fungal chitosanase in plant pathogenicity is now being done under progress.

CONCLUSION

Our experimental results showed that the N174 chitosanase gene can be superexpressed in *S. lividans*. The production media contain chitosan or natural chitosan compounds as carbon source. In early culture stages, we could obtain nearly pure chitosanase protein. This enzyme was collected and purified easily, which is significant in large scale industrial production. Such chitosanase quantities will allow further studies on crystallography, enzyme immobilization and structure-function relationship determination by chemical modification, limited proteolysis, terminal deletions etc. *Streptomyces* offers some important potential advantages over *E. coli* for the expression and the secretion of some proteins.

Current efforts are aimed at both increasing the capacity for heterologous gene expression in *Streptomyces* through the use of strong promoters and stabilizing the gene products by controlling protease expression. Our results showed that the high copy-number vector pFD666 is very useful for the expression of a cloned gene. The optimization of fermentation conditions can lead to important improvements in the production of proteins by *Streptomyces*. We hope that it would be possible to increase production levels or expand the range of proteins expressed by *Streptomyces* by defining the role that nutrient limitation and

the growth stage have on protease expression.

Although the high-molecular-weight chitosan oligomers, which were prepared by chitosanase hydrolysis have shown clearly antifungal activity and ability to induce mechanisms of plant resistance, there are still some problems related to the applicability of chitosan in practice. The reproducibility of these activities was not satisfactory. The use of these products in plant protection programs has to be evaluated. We do not know clearly what happens in plant cells between the time at which chitosan oligomers are applied and the time at which new mRNAs appear. Particularly, a better knowledge of the way by which chitosan is transported into the cells and the way it interacts with the cell receptors, would open the possibility of developing efficient methods of plant protection against fungal infection.

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